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UV-IRRADIATION SENSITIVITY AND MUTATION PRODUCTION IN THE HAPLOID SPORIDIA OF USTILAGO HORDEI

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "UV-irradiation sensitivity and mutation production in the haploid sporidia of Ustilago hordei," submitted by Cornelius Henry Hood, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Synchronized sporidial cultures of Ustilago hordei were irradiated with monochromatic ultraviolet light (2537 Å). Survival was plotted logarithmically against UV-dosage for cultures irradiated at 1/8 and 1/4 hour intervals throughout the cell cycle. The application of multi-hit and multi-target models of survival is discussed.

The survival curve may be characterized by two parameters, Do-value (a measure of slope) and extrapolation number (a measure of the extent of shoulder). Extrapolation numbers are high during the S, and low in the G_2 period. Do-values vary inversely with extrapolation numbers.

Low mutation rates during the cell cycle correspond to the period of rising extrapolation numbers. This is also the period in which irradiation produces a minimum of delay in the division cycle. It corresponds to the time of nuclear division and the early stages of DNA synthesis. High mutation rates occurred toward the end of the DNA synthetic period and in the beginning of the G_2 period.

At least two factors appear to influence survival; one, the over all sensitivity of the DNA, which may be due to structural changes in the DNA throughout the cell cycle; and two, the operation of repair mechanisms.

A total of 515 nutritionally deficient mutants were recovered and classified. Segregation studies on sporidia isolated from heterozygous teliospores of 82 mutant-by-normal crosses have revealed two cases in which the mutant loci are linked with the mating locus.

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INTRODUCTION

The suitability of *Ustilago hordei* (Pers.) Lagerh. as an organism for genetic study has been briefly discussed by Thomas (1965). Upon germination, the diploid teliospores undergo meiosis and produce a four-celled promycelium from which it is possible to separate the four meiotic products (sporidia) in an ordered sequence. The individual sporidia divide by means of budding, and sporidial cultures can be established and maintained indefinitely in axenic cultures. Since individual sporidia may be isolated, either by micromanipulations or by random-plating techniques, and cultured *in vitro* for indefinite periods before being mated to produce specific infective dikaryons, the investigator is afforded a considerable degree of genetic control in synthesizing parasitic strains, and in bringing these into combination with different genotypes of the host (see Perkins, 1949, for a similar discussion of *U. maydis*)(see Appendix 1 for life cycle of *U. hordei*).

Viewed as a system for general biological study, the characteristics of sporidial growth in *U. hordei* are such that standard bacteriological techniques can often be followed: the sporidia can be cultured on agar plates or slants, or they may be grown in liquid media; they can be plated and analyzed using macrocolony techniques; and they can be subjected to a variety of external influences under controlled conditions in such a way that the responses can be measured at both the cellular and the population level. It seems probable that *U. hordei* will lend itself well to biochemical analysis, and it may also prove well suited to studies in protein, RNA and DNA synthesis as well as for studies of a number of other physiological processes. Finally, *U. hordei* has the outstanding feature that it can be brought into an intimate relationship with its host during the parasitic stages of its development.

At present there are no reports of the existence of nutritionally deficient mutants in U. hordei. Furthermore, very little genetic work has been undertaken with this organism. The establishment of biochemically deficient strains would greatly enhance the usefulness of U. hordei as an experimental organism.

An initial objective of this study was to produce nutritionally deficient mutants in U. hordei. During the early attempts to induce mutations it was found that the sporidial cultures of U. hordei could be readily synchronized, making it possible to also study the cellular responses to external influences using an entire population growing and dividing as a single cell. The study of irradiation sensitivity of synchronized cultures of U. hordei became a second major objective in this work.

In the early studies, directed only toward mutation induction, it had been found that x-ray dosages exceeding 150,000 roentgens were required to reduce survival of U. hordei sporidia to the low level appropriate for the recovery of mutants. Considered in relation to the time required for one complete division in synchronized cultures, the time required to deliver 150,000 roentgens was relatively long. Moreover, it was found that the same degree of kill could be accomplished during a shorter exposure to UV irradiation. Because of the ease of manipulating UV irradiation compared with other mutagenic agents (including chemicals) it was decided to study synchronized cultures of U. hordei in relation to their sensitivity to UV irradiation and, at the same time, to study the UV treated populations from the point of view of mutation induction.

LITERATURE REVIEW

As pointed out by Zimmer (1961) the "hit" theory of Dessauer (Dessauer, 1922, 1931, 1933, 1954 cited in Zimmer, 1961) offered an explanation of the dose-effect curve in radiobiology, while the target theory (Crowther, 1924, 1926, 1927 cited in Zimmer, 1961; Crowther, 1938) provided an explanation for the fact that irradiation is so effective in inducing biological action. Both theories have undergone extensive critical analysis since they were proposed. In themselves these theories do not completely explain the phenomena revealed by radiobiological research; however, along with other concepts these theories have enabled the elucidation of the radiation effects on living cells (Lea, 1955) and have provided an understanding of the cellular response to irradiation effect. This knowledge has become extremely important in radiology and cancer treatment (Puck and Marcus, 1956; Hewitt and Wilson, 1959).

Though the "hit" theory and "target" theory had for a time been regarded as "old stuff" recent theoretical considerations have revived them. With modification they are being reapplied to biological investigation. Alper et al. (1960) suggested that extrapolation numbers be used and referred to as a "target" or "hit" number only when identification can be made with a cellular multiplicity of some sort. Do-values were used by Lajtha and Oliver (1961) in the sense of a "37% dose slope." This concept was extended by Alper et al. (1962) where the Do-value was defined as "that increment of dose which reduces the surviving fraction f to $f \cdot e^{-1}$ (or 0.37 f), once the survival curve has become exponential." The sigmoidal type of survival curve was discussed by Atwood and Norman

(1949), and it was proposed that this curve was best interpreted on the basis of a multi-unit, single-hit per unit hypothesis. More recently much consideration has been given to the theoretical aspects of survival-curve slopes and the differences that exist between multi-target and multi-hit models (Oliver and Shepstone, 1964; Fowler, 1964). The multi-target model refers to the "type C" curve which is characterized by a shoulder followed by an exponential region in the curve. The concept of extrapolation numbers has meaning only with this type of curve (Whitmore and Till, 1964). The widespread application of extrapolation numbers has been questioned by Barendsen et al. (1960). Their mammalian cell survival curves more appropriately fitted the multi-hit model. In their model the extrapolation number continues to increase as the survival curve is pushed to lower limits of survival.

It was discovered in 1949 that UV-irradiation damage could be repaired by photoreactivation (Kelner, 1949; Novick and Szilard, 1949; review in Hollaender, 1954). Setlow and Setlow (1962) showed that a large portion of the biological damage produced by UV irradiation was due to thymine dimerization. Shortly thereafter it was discovered that yeast contained an enzyme that stimulated photoreactivation repair (Rupert, 1960).

The discovery of radiosensitive mutants of Escherichia coli (Hill's strain - E. coli B_{s-1}, Hill, 1958; Hill and Simpson, 1961) along with the fact that enzymatic photoreactivation repair could break dimers (Wulff and Rupert, 1962; Setlow and Setlow, 1963) has strengthened the concept that DNA contains the information to govern its own repair. Comparative studies on normal and UV-sensitive strains of E. coli suggested that UV sensitivity was a consequence of the inability to

repair UV damage. This repair mechanism (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964) involved the excision of dimers as oligotide fractions, which was then followed by replacement of the nucleotide sequence. Howard-Flanders and Boyce (1965) have mapped three loci which are involved in the genetic control of repair phenomena in E. coli K12.

The nature of the repair process was studied from a different aspect by Witkin (1958, 1963a) when she established that "dark repair" occurred in E. coli under conditions of amino-acid deprivation following UV irradiation. She also showed (Witkin, 1961, 1963b) that the repair process could be prevented by treating irradiated bacteria with either caffeine or acriflavine dye. The work of Doudney (1963) substantiated the observations of Witkin, that: (i) following UV irradiation, mutations declined in the absence of protein and RNA synthesis; and (ii) DNA replication must occur in order that a mutation be fixed into the genome. More recent work by Doudney (1965) indicated that caffeine and acriflavine dye inhibited DNA synthesis in irradiated E. coli but did not do so in non-irradiated bacteria. The implication was that these two chemical compounds combined with the irradiation-lesion and thus prevented its repair and replication. This would offer an explanation for Witkin's earlier observation.

Repair mechanisms have been invoked to explain part of the changing pattern of X-ray sensitivity throughout the cell-cycle of mammalian cells. Using fractionated X-ray treatment Elkind and Sutton (1959) showed that repair of radiation damage occurred and that the degree of repair was to a large extent dependent on the length of the

interval between the two doses. Using a synchronized cell system Terasima and Tolmach (1963a, 1963b) and Tolmach, Terasima and Phillips (1965) have revealed a changing pattern of X-ray sensitivity which varied as a function of cell age in the DNA synthetic cycle. Using the terminology proposed for different stages of the cell cycle by Howard and Pelc (1953), these authors found that maximum sensitivity occurred in the late G_1 and early S periods. They proposed that irradiation damage is repaired when it occurs early in the G_1 period; however, the more the cells advance into G_1 the shorter is the period for repair before the cells reach the critical DNA synthetic period. According to the hypothesis " -- completeness of repair would be inversely related to the number of abnormalities transferred to newly-formed DNA, and, therefore to the probability that the cell or its progeny will not be able to reproduce."

Survival curves obtained through X-irradiation of different aged cultures of L-cells were analyzed by Whitmore and Till (1965) in terms of Do-values and extrapolation numbers. Extrapolation numbers rose during the S-period and declined during the G_2-period to a low during G_1; an inverse relationship occurred for Do-values.

Another aspect of a cell's response to X-irradiation is division delay. Terasima and Tolmach (1963) reported that cells in G_1 show a minimal delay; thereafter the delay increases progressively until just before the next mitosis, and then declines.

Three studies involving UV-irradiation have related UV sensitivity to aspects of the cell cycle. Iverson and Giese (1956) were able to correlate UV sensitivity to the amount of DNA present in Tetrahymena cells. With high DNA content UV resistance was high; the resistance dropped at mitosis when DNA per cell was reduced. Holliday

(1964) working with U. maydis established that cells were resistant during non-DNA synthetic periods. Resistance decreased during mitosis to the most sensitive point which was presumed to be the point where all cells were synthesizing DNA. Swan (1962) observed essentially the same pattern of UV sensitivity in Schizosaccharomyces pombe.

Several studies were carried out on the forward and reverse mutation rates of Ustilago maydis by Ishikawa (1956, 1957, 1962). He made extensive use of an unstable prototrophic strain which had a high spontaneous mutation rate to homocystineless auxotrophs. A study of both the mutants and revertants of the mutants revealed that they were not all unstable. He was able to distinguish three distinct groups of alleles on the basis of their reverse mutation rates.

MATERIALS AND METHODS

1. Biological Material

Monosporidial lines of U. hordei were provided by Mr. P. Thomas. Of these lines, $I_4(+)$ and $E_3(-)$ were used in irradiation studies and mutation production; mutants of $I_4(+)$ and $E_3(-)$ were mated with $I_1(-)$ and $E_1(+)$ respectively in order to carry them through the infective stages on the barley host. Vantage and Hannchen, two varieties which are highly susceptible to infection from crosses between these lines, were used as hosts to carry the U. hordei mutants through the diploid phase (Thomas 1965).

2. Medium

A modified Vogel's (1956) medium was used. Complete medium contained: 5 g Difco yeast extract, 5 g salt-free caseine hydrolysate (N.B. Co.), 10 g dextrose, 50 mg tryptophane, 20 ml Vogel's salt solution, 10 ml vitamin solution (Holliday 1961), 1 litre distilled water. For solid medium 1.7% Difco bacto agar was added for agar plates and 2.5% for agar slants.

Minimal medium contained 20 ml Vogel's salt solution and 10 g dextrose per litre of distilled water.

Supplemented minimal medium was prepared according to Holliday (1961). The individual growth factors were added to minimal medium as required: amino acids 100 mg, purines and pyrimidines 10 mg, and vitamins 1 mg per litre.

3. Growth and Synchronization

Sporidial cultures were grown in complete medium and maintained at a constant temperature in a New Brunswick incubator-shaker Model R27. The cultures were grown in 500 ml DeLong culture flasks, fitted with Morton stainless steel closures and containing 150 ml of complete medium. In order to establish the optimum growing temperatures, growth curves were determined over a range of temperatures from 12° C to 32° C. Cell concentrations were determined by making periodic counts with a hemacytometer counting chamber (Spencer with improved Neubauer ruling).

A constant temperature of 22°C was used for growth and synchronization of cultures. Early investigation showed that cultures could be readily synchronized. In subsequent experimental work, two procedures were used in establishing synchrony.

(i) Procedure A

It was found that cultures which are maintained by transferring 1 ml of sample to fresh 150 ml flasks every 48 hours will be in a condition of synchronized division at the time of making each transfer. To make an experimental culture one merely adjusts the size of aliquot transferred from the 48-hour culture. Experimental cultures were established by transferring 10 ml of material from a 48-hour culture (approximate concentration of 1.1×10^8 cells/ml) to a fresh culture bottle containing 150 ml of medium. This produced a synchronously dividing culture with an approximate cell concentration of 7.0 to 10^6. One-tenth ml aliquots were periodically removed from

the newly established cultures and diluted in 10 ml of cold water (4^{o} C). Controls were plated following a dilution of 1 in 50. A second 1 ml sample was irradiated by monochromatic ultraviolet light with a wave length of 2537 $\overset{o}{A}$ngstrom units.

(ii) Procedure B

An alternative technique was also used in preparing cells for synchronized division. This method involved the reduction of agitation of a logarithmically growing culture when the cell concentration was between 7.5×10^{7} and 8.0×10^{7}/ml. This was accomplished by transferring 15 ml of culture from the DeLong flask to a sterile 50 ml Erlenmeyer flask which was then fixed in position on the same shaker. The restricted volume of the smaller flask caused a reduction in agitation, which was maintained for a 17 to 18 hour period (overnight). Culture material thus treated was used for experimental studies as indicated above for procedure A.

Irradiated material was either plated directly or with appropriate dilution depending on the amount of ultraviolet irradiation applied. Dilution tubes and pipettes were pre-cooled to 4^{o} C in order to avoid excessive temperature fluctuations during the experimental period. Following plating of irradiated material on complete medium, the agar plates were incubated at a constant temperature of 22^{o} C for 4 to 5 days.

4. Radiation Procedure

During exploratory work both X-ray and UV irradiation were employed. X-ray irradiation was delivered by a Philips MG 300 X-ray

machine which was operated at a potential of 300 kv and 10 ma. Dosimetry was carried out using a Philips Universal Dosimeter. The distance from the tube face was adjusted to give a dose rate of 750 r per minute. Before treatment the sporidia were centrifuged and re-suspended in water at approximately 2.0 a 10^6 cells per ml; 2 ml samples were then placed in small glass vials 15 mm deep and 22 mm in diameter and located in the centre of the X-ray target area; 0.1 ml samples were withdrawn and diluted as desired for dose-effect studies and mutation selection.

Early UV irradiation was carried out on 2 ml samples prepared in the same way as material prepared for X-ray irradiation. UV irradiation was delivered by a General Electric 15 watt germicidal lamp 68 cm distance from the sample which was placed in a 5 cm Petri dish and constantly agitated. Sampling and plating were carried out at appropriate intervals.

The UV dosage delivered at the source was measured using a Westinghouse ultraviolet meter SM 200 which was fitted with phototube WL-773 thorium cathode. Maximum tube response occurred at 2550 Angstrom units with a response range from 2000 to 3675 Angstrom units.

With the establishment of synchronization of sporidial division in shake cultures it was imperative that a better controlled system be developed in order to study irradiation effects over the 2-1/2 hour synchronized cell cycle. An ultraviolet light irradiating system was developed for this purpose.

A 50 watt Hanovia low-pressure mercury vapor "U" lamp was used as an ultraviolet light source. The lamp was mounted vertically in a cylindrical tube which was fitted below with a Baird Atomic 2" x 2" interference filter 2537Å. 1 ml samples to be irradiated were placed in small vials 22 mm in diameter and 10 mm deep which were cooled by an ice bath contained in a plastic Petri dish 100 x 15 mm. The sample in its ice bath was then placed on a Canlab magnetic stirrer No. 77-881/1250 so that the sample was centered beneath the interference filter at a distance precisely 16 mm from it. Sample agitation was controlled by means of a stainless steel pin placed in the vial.

Samples were irradiated at a rate of 630 ergs/mm^2/min to give survivals ranging from 0.5% to 1.0%.

5. Replica Plating and Mutant Selection

When the colonies that developed from irradiated sporidia had reached 1 to 1.5 mm in diameter, they were replicated on minimal medium by the technique of Lederberg and Lederberg (1952). Following a second period of incubation of 2 to 3 days, those colonies which had not developed on minimal medium were removed with a sterile needle and spotted on both complete and minimal agar plates. After a 4 to 5 day incubation period those selections that developed well on complete and showed little or no development on minimal were placed on complete agar slants for storage and for later study. These were subcultured to new slants at three month intervals.

6. Mutant Identification and Auxanographic Studies

The method of Holliday (1961) was used to characterize the mutants with respect to nutritional deficiency. Following the initial identification all mutants were tested auxanographically by spreading a water suspension of the sporidia on minimal plates containing the individual nutrients.

7. Mating and Host Inoculation

Essentially, the methods described and used by Mr. P. Thomas (1965) were followed in preparing compatible sporidial cultures and inoculating these on the barley seed. Since only one or two crosses were made with each mated pair, smaller quantities of complete medium were used. 125 ml Erlenmeyer flasks containing 25 to 50 ml of complete medium were inoculated with fresh sporidial material taken from slants, plates or liquid cultures. Erlenmeyer flasks were capped with aluminum foil to prevent contamination. Following the mating of sporidial mutant strains, the bottles were allowed to shake at room temperature for 24 hours. An estimated 100 formaldehyde-treated barley seeds in three-dram vials were covered with the inoculum. Following a vacuum treatment of 20 minutes at 20 inches of mercury the excess of inoculum was poured off. The seeds were placed in a coin envelope and left to dry in a well aerated environment for 5 to 10 days.

8. Growth of the Infected Barley Host

Five different sowings were carried out, two in the University growth chambers and three in the field of the University

farm. The teliospores obtained from these sowings, with the exception of the field crop of the present year, have been packeted and stored.

9. Segregation and Recovery of Mutants from Teliospores

Two methods may be used in segregating the four meiotic products that develop from a germinated teliospore. One, using a de-Fonbrune micromanipulator (Dickinson 1926), though time consuming, must be employed in order to recover the four segregating sporidia of a single teliospore. As the objective at this stage was to recover mutants, especially in recombination with the opposite mating-type locus, a technique of random recovery of mutant types was used. A dilute suspension of teliospores in complete growth medium was prepared by aseptic technique. Teliospores were removed from infected barley spikes to alcohol-sterilized paper with a sharp sterile instrument and then introduced into 30 to 50 ml of complete medium in 125 ml Erlenmeyer flasks. Sporidia produced by germinating teliospores readily disperse in the shake culture. When the appropriate concentration of sporidia was reached (between 10^6 and 10^7/ml) they were plated on complete agar plates. A hemacytometer count on the culture was followed by appropriate dilution to provide between 50 and 105 colonies per plate.

Plates were incubated 4 to 5 days at 22° C and then replicated on minimal medium. Two to three days further incubation revealed the nutritionally-deficient colonies. With a sterile needle several of these deficient colonies were removed, and each streaked

on complete and minimal plates. After some development of sporidial material the mating-type of each isolate was determined by means of the Bauch test (Bauch 1927, 1932, cited in Fischer and Holton 1957). Isolates of opposite mating types were then transferred to agar slants.

Generally, spores removed from smutted heads harvested from a growth-chamber crop are free of contaminants. Field-grown material is usually heavily contaminated by other organisms, especially bacteria. The bacteria were satisfactorily inhibited, with no apparent ill effect on teliospore germination and sporidial growth, by the addition of a streptomycin-penicillin mixture at a rate of 50 units each per ml of culture medium.

10. Studies on Reverse Mutation

An unstable, slightly, leaky arginine mutant No. V-441 was used in reverse mutation studies. The evidence now available suggests that both protein and DNA synthesis are required for the fixation of mutants into the genetic material. Therefore, if a potential reverse mutant is to become established on minimal medium it must be supplied with small quantities of nutrients for which it was deficient or it must be capable of some growth on its own (i.e. it is leaky). Mutant V-441 was used because it was able to grow very slowly on minimal medium. The instability of the mutant made it possible to obtain significant data with a small amount of experimental work.

The mutant could be readily synchronized by techniques described earlier. To establish experimental cultures, 15 ml of saturated sporidial culture that had been exposed to synchronization

procedures were centrifuged for five minutes at 1500 r.p.m. The stale medium along with a small percentage of uncentrifuged cells was poured off and the remaining cells diluted into 25 ml of complete medium in a 125 ml Erlenmeyer flask which was then incubated at 22° C in an incubator shaker.

Samples for UV irradiation were prepared by removing one ml aliquots periodically and diluting them in 10 ml cold water. This provided samples with an approximate concentration of 1.2×10^6 cells per ml. Controls were plated on complete medium after appropriate dilution. A plating was also made with nonirradiated material on minimal plates at an 100-fold concentration (1.2×10^4 sporidia per plate) to establish the spontaneous reverse mutation rate. Irradiated material was plated directly on minimal plates (0.1 ml per plate) to establish the effect of UV irradiation on reverse mutation. After dilution, additional irradiated material was plated on complete plates to determine the percentage survival.

11. Staining and Photography

A modified commercial, cotton blue, lacto-phenol stain containing 0.8 to 1% cotton-blue in equal quantities of lactic acid, phenol, glycerine and water was used. Glycerine was added to the commercial stain to increase the volume by 20%.

Photography was done on a Leitz microscope fitted with an apochromatic objective 40/0.95 N.A. using a Leitz Aristophot 4 x 5 inch camera. Representative fields were photographed on Panatomic-X film.

RESULTS

1. Growth

Development in the haploid phase of U. hordei ranges from a condition of complete sporidial production to almost entirely mycelial growth depending on environmental conditions. As one major objective in this study was to irradiate sporidial cultures and to plate them for colony survival and mutant selection, it was imperative that the cultures be maintained in a condition which encouraged sporidial rather than mycelial growth. Sporidial cells are characteristically uninucleate and fairly uniform in size whereas the nuclear content of the more variable mycelia is somewhat ambiguous, presenting problems both with respect to experimental manipulation and in the selection of mutants.

The studies on growth of U. hordei sporidia in liquid culture were designed to determine the optimum growing conditions in culture, as well as to elucidate environmental factors that encourage sporidial growth. All cultures on which growth studies were made were initiated as subcultures of a log-growing parent culture at the same temperature. To avoid irregularities that might occur in newly started cultures, no data were recorded until several hours after the initiation of new cultures. All cultures were grown in complete medium throughout the study. It was established early in these studies that although the sporidia grow successfully in minimal medium they very readily become mycelial in it. Minimal medium was

therefore considered unsuitable for growing cultures that were to be used in irradiation studies.

Fig. 1 is representative of the type of information obtained on sporidial growth. It shows the growth patterns of U. hordei sporidia at 22° C, the temperature at which all irradiation studies were carried out. The generation time over the period of logarithmic growth is 5.0 hours. This is the time during which the cell concentration doubles. Following a logarithmic phase, the cells cease budding, enlarge somewhat and become less vacuolated (compare 0-hour cultures with logarithmic cultures, Plates 1 and 2). The concentrations of cells at the end of logarithmic phase is about 1.2×10^8 per ml. During the next 20 hours in which no cell division is apparent, there is a gradual increase in the number of doublet cells. These cells have a slight constriction at the midregion, perpendicular to the long axis. In cells stained with cotton-blue, a cross wall is quite evident (see 0-hour and 1-3/4 hour photographs Plate 1). Sporidia in cultures which are maintained in normal growing conditions for an extended period germinate to produce a secondary mycelial growth.

Growth of two sporidial lines, $E_3(-)$ and $I_4(+)$, was studied over a range of temperature from 12° C to 32° C. The relation between multiplication rate and temperature is expressed in Fig. 2. The optimum multiplication rate occurs at 25° C. In addition to changes in the rate of division of the sporidia at various temperatures, certain other pertinent variations in growth response were noted.

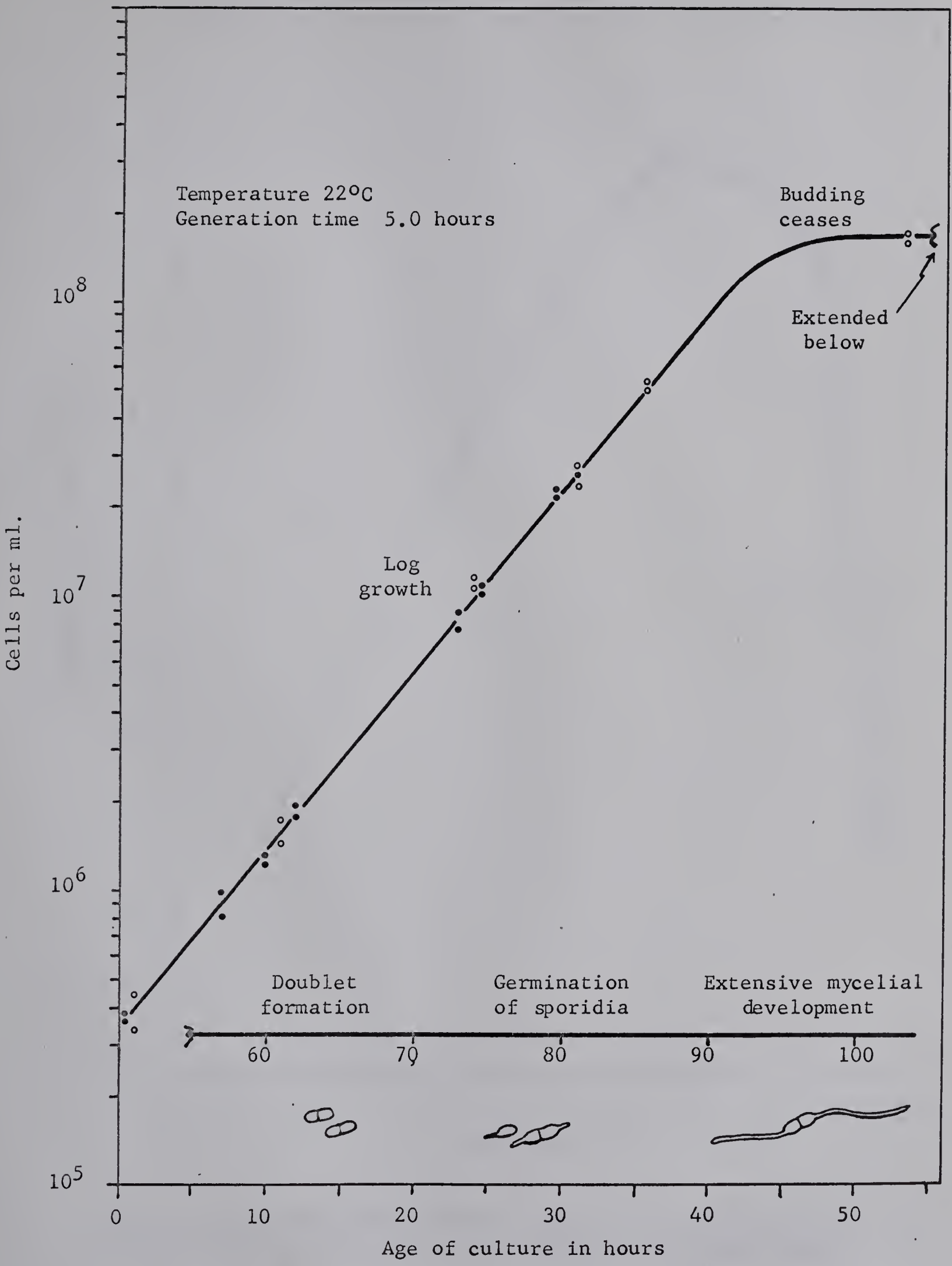


Fig. 1. Relationship between growth and age of culture in Ustilago hordei .

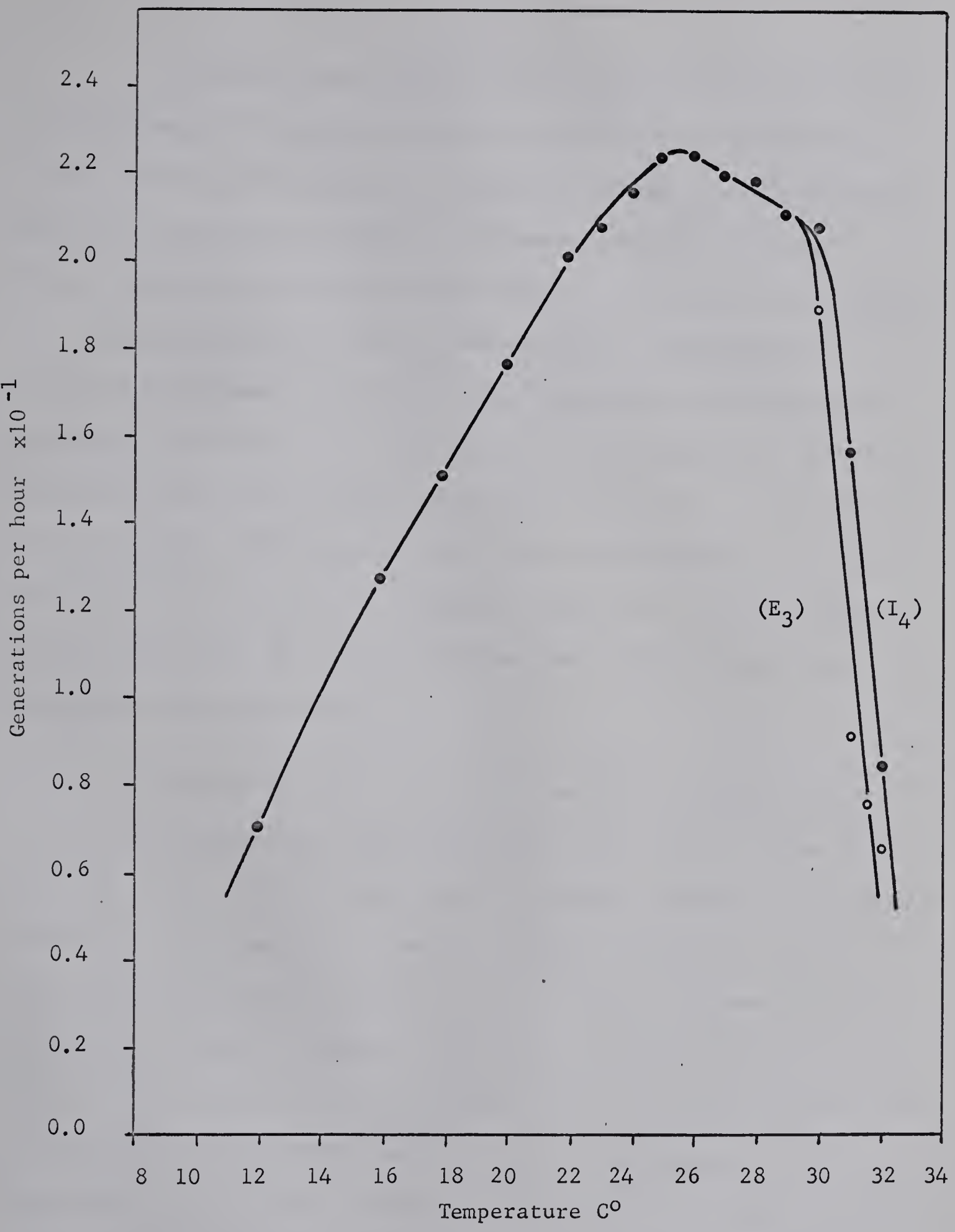


Fig. 2. Relation between multiplication rate and temperature in two monosporidial lines (E_3 and I_4--P. Thomas) of Ustilago hordei.

As higher temperatures of 27° C to 29° C were reached the sporidia tended to remain attached in small clusters, especially toward the end of the logarithmic phase of growth. Mycelial development also occurred more readily. At temperatures of 30° C and higher, sporidial development became quite irregular. Large fragments and irregular sporidial clusters were produced. The density of cellular development at the end of the logarithmic phase was much reduced (in the order of 7×10^7 sporidial units per ml.). At 31° C and 32° C many cells developed very heavy cell walls and appeared to cease dividing. Occasionally one of these heavy-walled spore-like cells was seen to produce an irregular sporidium. At these temperatures, differences in the rate of growth were noted for the two sporidial cultures studied.

Between 20° C and 25° C there was little apparent change in sporidial morphology as the temperature was lowered. Below 20° C the size of the sporidia was reduced and their morphology was altered. Sporidia became rounded and somewhat smaller, and tended to remain clustered in small packets. The union did not appear to involve cell wall material but rather a mucilaginous coat which held the sporidia in close proximity for an extended period. At lower temperatures the sporidia did not cease budding as the concentration of cells approached 1.2×10^8/ml. Concentrations as high as 4.0 and 5.0 x 10^8 cells/ml. have been recorded at 18° C. It was also noted that mycelial development did not occur at these lower temperatures.

These features of sporidial growth in response to environmental changes parallel observations recorded for spore germination by Kniep (1926, cited in Nielsen 1965) in which spore germination in U. hordei was essentially of mycelial type on water agar, yet was sporidial in a nutrient medium. Hüttig (1931, cited in Nielsen 1965) indicated the same effect for germination at higher temperatures (25°C to 30° C). By using low-temperature germination, Thren (1937) was able to produce a semblance of sporidial germination in the otherwise mycelial germinating spores of U. nuda.

2. Synchronization

In early studies using X-ray irradiation, extreme variations in radiation sensitivity were observed from one experiment to the next. A study to clarify the reasons for these changes in sensitivity indicated that regular fluctuations in radiation sensitivity occurred during early stages of growth of fresh cultures initiated from non-budding sporidia. Numerous cell counts on further cultures, prepared in the same way, revealed that a high degree of synchrony could be obtained if the period from cessation of budding to the time of re-inoculation to new medium was carefully controlled. Later investigations showed that sporidial cultures in logarithmic phase with cell concentrations of 7.5 to 8.0 x 10^7/ml., would stop dividing if the rate of agitation of the culture was reduced for a 17 to 18-hour period.

Cultures synchronized by the former method (Procedure A) usually contained a small fraction of sporidia with short mycelial

projections. These are not present in cultures prepared by the second method (Procedure B). The latter are more sensitive to environmental influences, particularly to changes in agitation during the synchronization period. Considerable care must be taken to have proper concentrations of sporidia before beginning to reduce the rate of agitation.

In type A cultures, synchronous bursts are distinct, but colony counts do not show a doubling of cells with each divison (See Appendix 2 and Fig. 8.). Cell-increase at each division is somewhat higher when determined by hemacytometer counts. This suggests that the daughter cells are less vigorous than are the parent cells and that they suffer a higher death rate during dilution and plating. Cell counts on type B cultures indicate that cell numbers double with each synchronous division (See Appendix 3 and Fig. 9). Although not doubled, the plating counts show a much higher increase than type A cultures with each mitotic burst, 80% to 90% as compared with 55% to 70%.

Plates I and II show sporidia photographed at different stages of synchronized cell division. They are representative of the characteristics of synchronization that are obtained in cultures prepared by procedure B. Note that sporidia freshly transferred to new medium (0 hr) are much less vacuolated than are the actively dividing sporidia. Sporidia in the first two division cycles are larger than those in logarithmic cultures. Doublet cells can be seen in the zero to 1-3/4 hour photographs; these usually divide more slowly than do the singles. The number of doublets can be kept to a minimum by controlling the length of time a culture is left in the so called stationary

Plate 1 Sporidia photographed at different stages in cycle one of synchronized cell division. Doublet sporidia are circled in photograph of culture ages 0 hr., 1 hr., and 1-3/4 hr. (Synchronization procedure B).

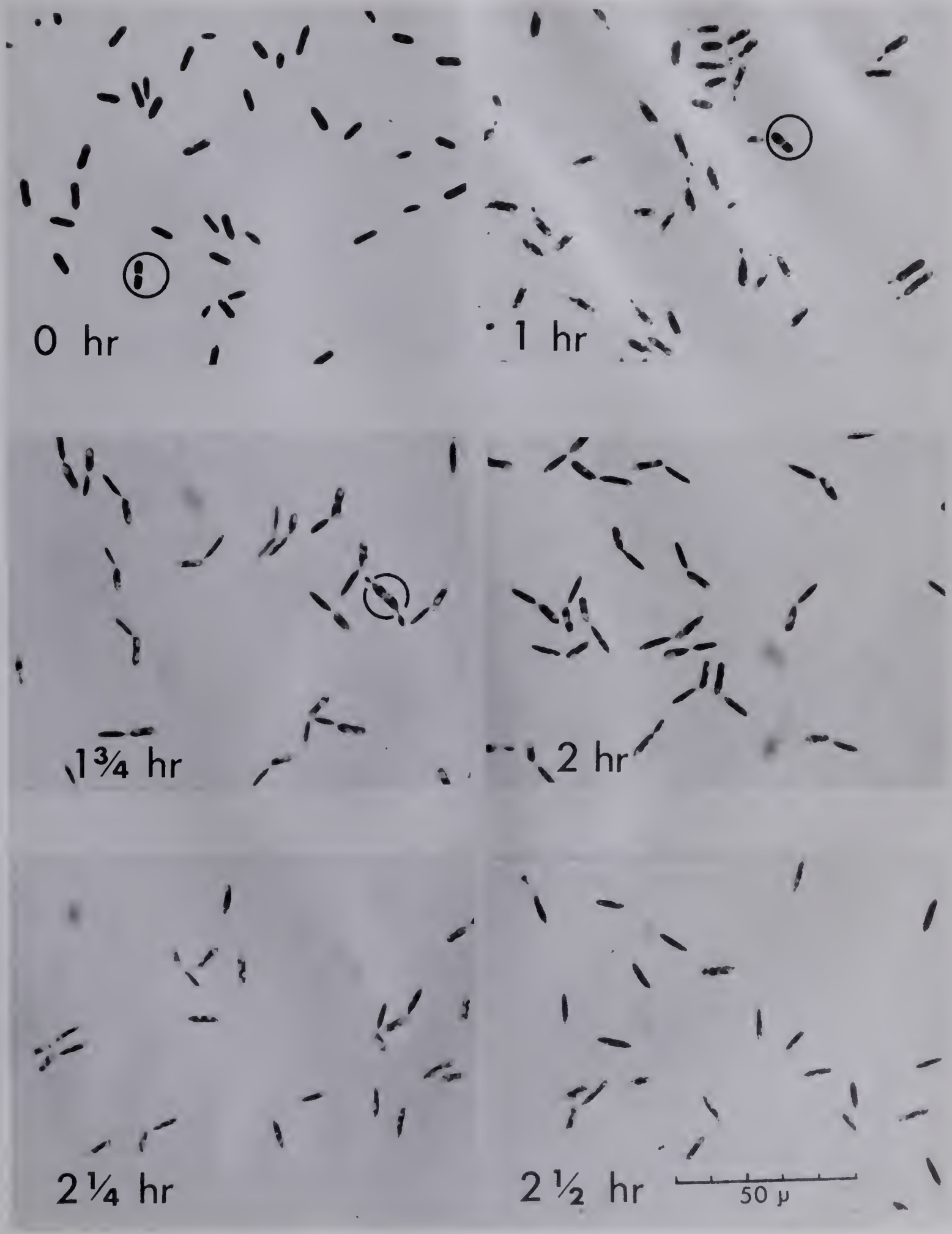
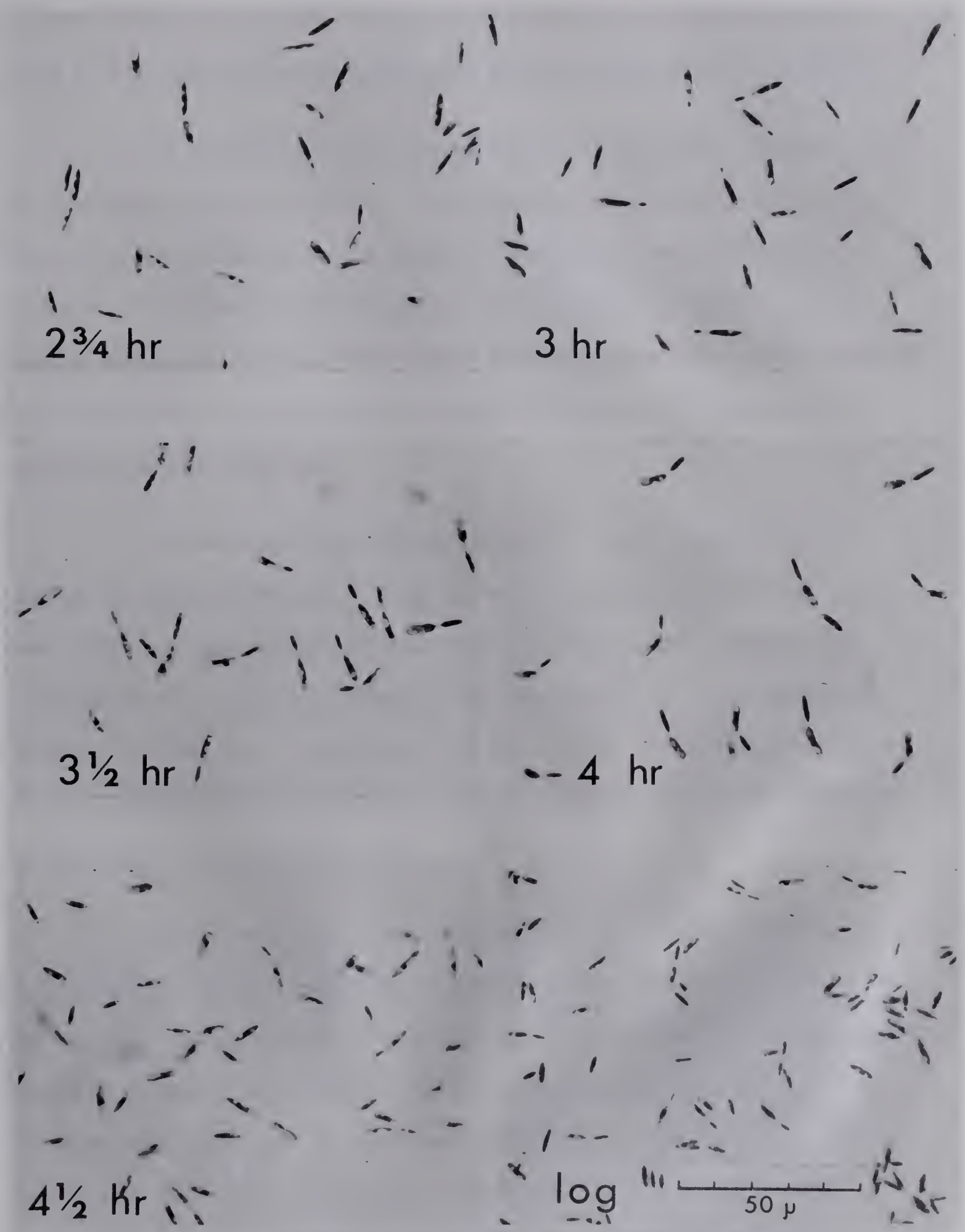

0 hr
1 hr
1¾ hr
2 hr
2¼ hr
2½ hr
50 μ

Plate II Sporidia photographed at different stages in cycle two of synchronized cell division (Synchronization procedure B). The photograph in the lower right corner shows sporidia in the logarithmic phase of growth at 22°C.


2¾ hr
3 hr
3½ hr
4 hr
4½ hr
log
50 μ

or non-budding state before they are transferred to fresh medium. Type B cultures generally have fewer doublets than type A cultures.

All synchronization and radiation studies were carried out with cultures grown at 22° C. This was done for three reasons: (i) there was good separation of daughter and parent cells following mitosis; (ii) mycelial development was more easily avoided than at higher temperatures; and (iii) the duration of the cell cycle was probably increased, thus providing a longer interval for experimental analysis of the cell cycle.

The method of Engelberg (196) was used to quantify the degree of synchronization. Figs. 3 and 4 present data for synchronized divisions <u>one</u> and <u>two</u> respectively. These graphs compare the normalized rate of cell division, $R = (dn/dt)/n$, in a synchronized culture to that of a logarithmic culture. R is the fractional increase in cell numbers per unit of time (See Appendices 4 and 5 for data on which Figs. 3 and 4 are based).

The percentage of synchronization for growth cycles <u>one</u> and <u>two</u> are 68% and 48% respectively. Most of the reduction in synchrony or "synchronization decay", arises from a differential in division rate between parental and daughter cells during the second cycle. It is noted that there is an increase in doubling time amounting to 10 minutes in division-cycle <u>two</u> as compared to division-cycle <u>one</u>. This increase is not surprising when it is considered that the doubling time of a log-dividing culture at 22° C is 300 minutes.

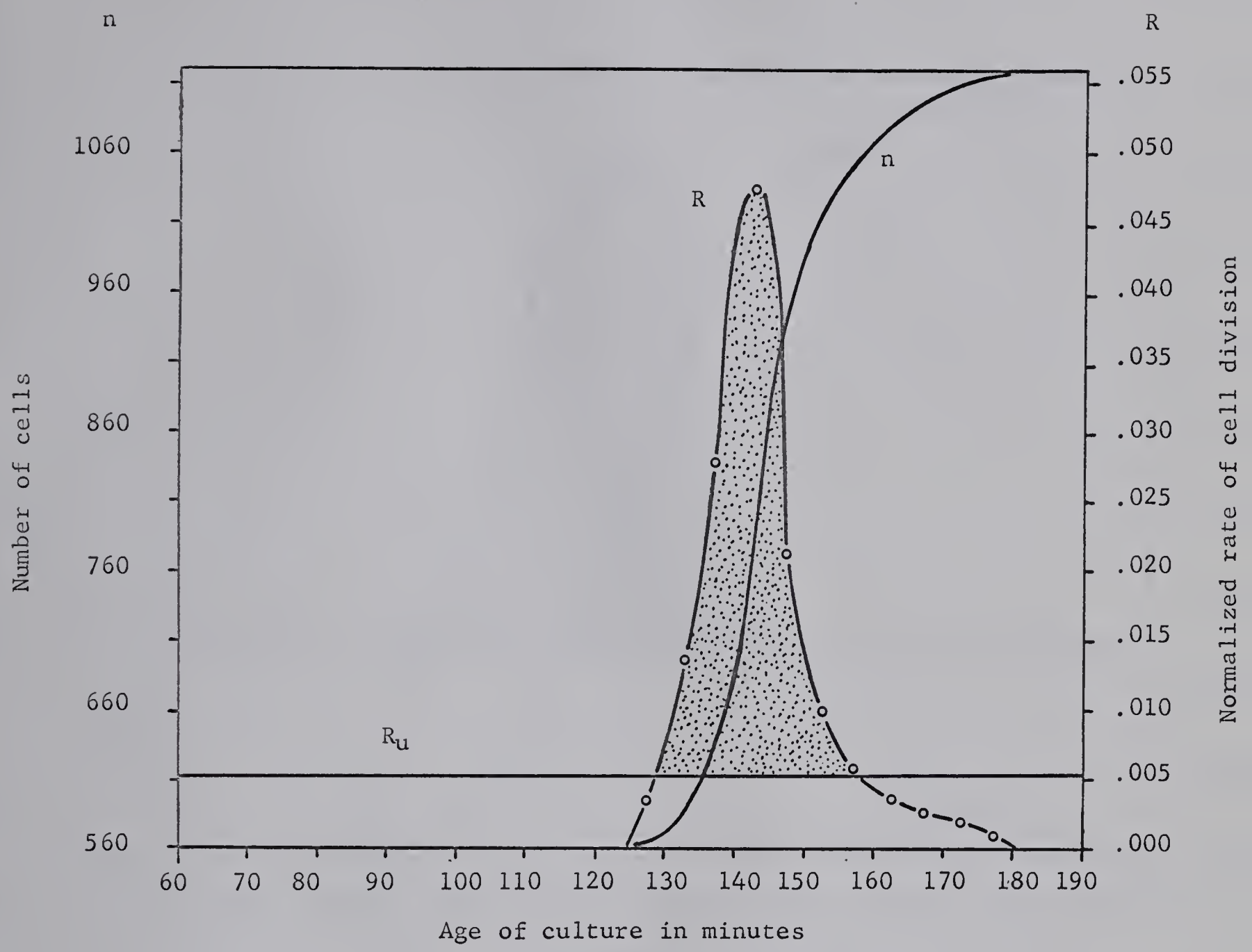


Fig. 3. Percentage synchronization (Method of Engelberg 1961) of synchronized division one, Ustilago hordei. R, Normalized rate of cell division $\left[R = (dn/dt)/n\right]$; Ru, normalized rate of cell division for a logarithmic culture having a doubling time of 130 minutes. Shaded area is the 'overlap area'. Percentage synchronization = 68 per cent.

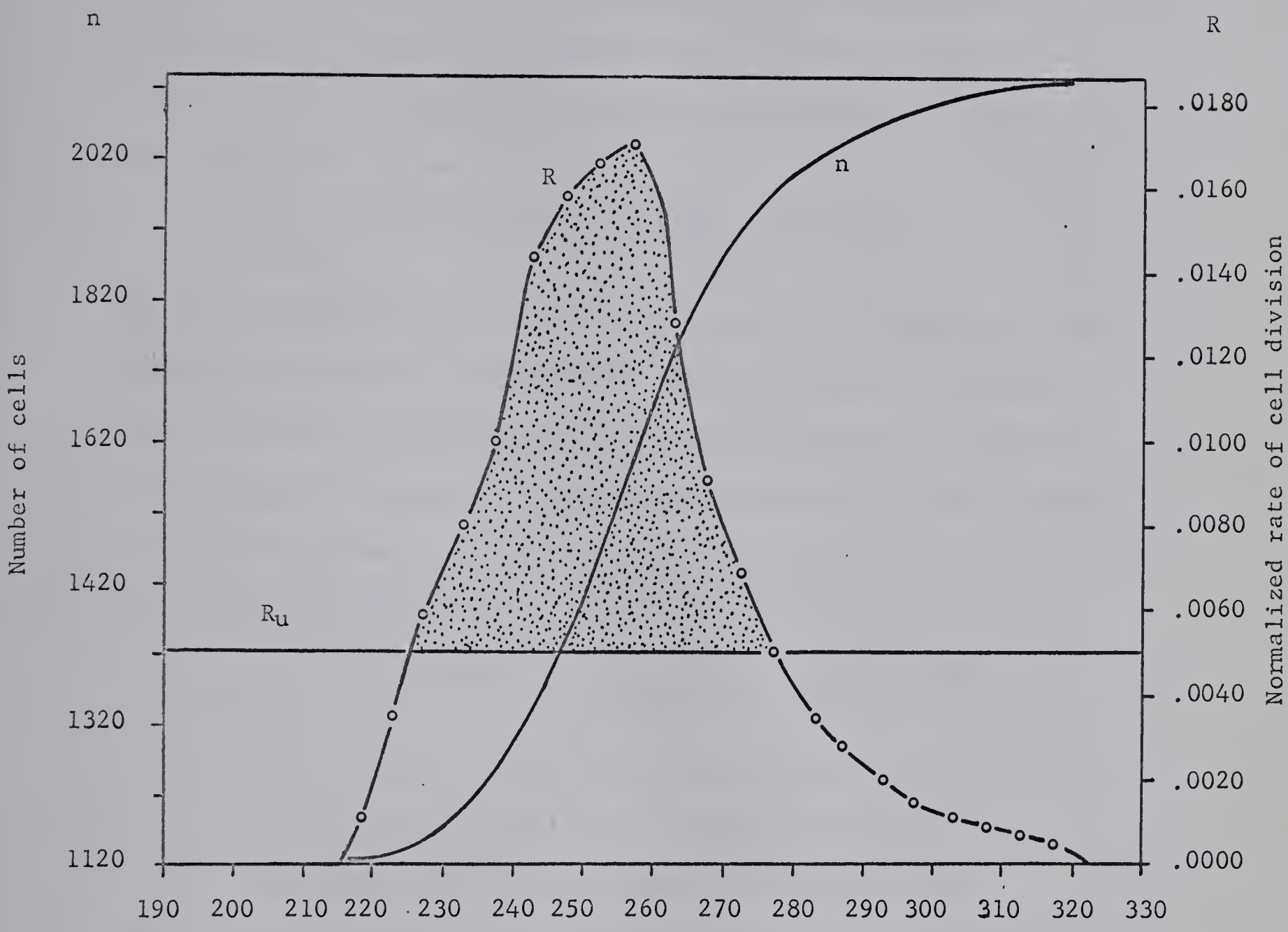


Fig. 4. Percentage synchronization (Method of Engelberg 1961) of synchronized division two, Ustilago hordei. R, Normalized rate of cell division [R = (dn/dt)/n] ; Ru, normalized rate of cell division for a logarithmic culture having a doubling time of 140 minutes. Shaded area is the 'overlap area'. Percentage synchronization = 48 per cent.

For the first cell division two additional methods were used to estimate the degree of synchronization. Zeuthen (1958) relates synchronization to the fraction of the generation time in which 50% of the population doubles:

$$\% \text{ synchronization} = \left(\frac{Tg/2 - T50}{Tg/2}\right), \text{ where}$$

Tg is the generation time and T50 (estimated to be 9 minutes) is the shortest time-interval during which 50% of the population doubles. Synchronization was estimated by this method to be 83%. Scherbaum (1959) measures the degree of synchrony in terms of (i) the fraction of the generation time, tg, in which synchronous division occurs, and (ii) the increase in the population over this time:

$$\% \text{ synchronization} = \left(\frac{n_2 - n_1}{n_1}\right)\left(1 - \frac{dt}{tg}\right), \text{ where}$$

n_1 = number of cells before multiplication (560)

n_2 = number of cells after multiplication (1075)

dt = time span of cell division in minutes (35)

The percentage synchronization as determined by this method was 67%.

3. Irradiation Studies and Mutation Rates

Early X-ray and UV-irradiation studies revealed,(1) that sporidia in the logarithmic phase of growth were more sensitive to both types of irradiation than were sporidia that had ceased budding and (2) that dividing sporidia became increasingly more sensitive from the time buds appeared until the daughter and parent sporidia separated. Thereafter sensitivity decreased until the next generation of buds appeared.

(i) Irradiation results using synchrony procedure A.

Twenty-two experiments were performed on monosporidial line $I_4(+)$, using an improved irradiating system (See Methods and Materials). Synchronization procedure A was used throughout experiments I to XVIII. Three preliminary experiments were performed to establish survival curves for cell culturesof different ages. The dosage of UV irradiation was then adjusted to provide survival rates of 0.5% to 10%. Two irradiation dosages were used for each culture-age treatment. Only the data obtained from experiments XIV to XVIII are included in Appendix 6.

When the information from the five experiments reported in Appendix 6 is combined, the number of data pairs range from 2 to 8 for each culture-age irradiation treatment. Where there are more than two pairs of data for each treatment the best fit for the survival curves was determined by calculating the regression coefficients (byx), and establishing regression lines (Snedecor, 1956, Johnson, 1963). Since the regression coefficients represent the change in the logarithm of the percentage survival (y) for each unit change in (x), it was a simple matter to convert these coefficients to Do values by using the formula (1/byx) (0.4318). Where there were only two data-pairs the slope of the line joining the two plots was considered to be the equivalent of a regression line.* The amount of change in y per unit change in x was calculated by simple division, and although the values obtained are not regression coefficients, they were handled as such in making conversions to Do values.

* It is hoped that the freedom used with the terminology does not unduly offend the statistician.

The "t" test was applied to those regression coefficients that were calculated from more than two data-pairs. They all exceeded the one percent level of significance. The significance of the difference between the regression coefficient pairs, 1-3/4 and 3-1/4 hours, and 3-1/4 and 3-3/4 hours exceeded the one percent level when the "t" test was applied to them.

Three representative survival curves (regression lines) are shown in Fig. 5. The symbols used represent data from three separate experiments; closed and open symbols are used to distinguish between different culture-age treatments. These appear to be "sigmoidal" or type "c" curves i.e. the curve is characterized by a shoulder followed by a region of exponential decline. The possibility that these are multi-hit curves is not excluded (Alper et al., 1962; Fowler, 1964).

Do values, defined as the dose required to reduce the surviving fraction, f, at any point on the linear portion of the survival curve to 0.37f, were calculated from the byx values. The extrapolation numbers were obtained by plotting survival curves and extrapolating the linear portion of these curves back to their intersection with the axis along which percentage survival was plotted. Do values and extrapolation numbers for data of experiments XIV to XVIII are plotted in Fig. 6.

The upper half of Fig. 6 shows Do values plotted as a function of cell age and the lower half shows extrapolation numbers. Time zero refers to the time of initiating the cultures. Sporidia begin to bud at 1 hour. Daughter cells separate from the parent cells between

Fig. 5. The relationship between dosage of UV irradiation in minutes (delivered at a rate of 630 ergs/mm^2/min) and the percentage of cells that survive UV treatment. The different symbols (circles, triangles and squares) represent data of three separate experiments (XIV, XV, and XVI respectively); different culture-age treatments are represented by open and closed symbols. The 1-3/4 hour curve is supported by data of two experiments only. Do values and extrapolation numbers are presented for each of the survival curves. The UV dosage was adjusted for each culture age treatment in order to provide a surviving fraction ranging between 0.5% and 10%.

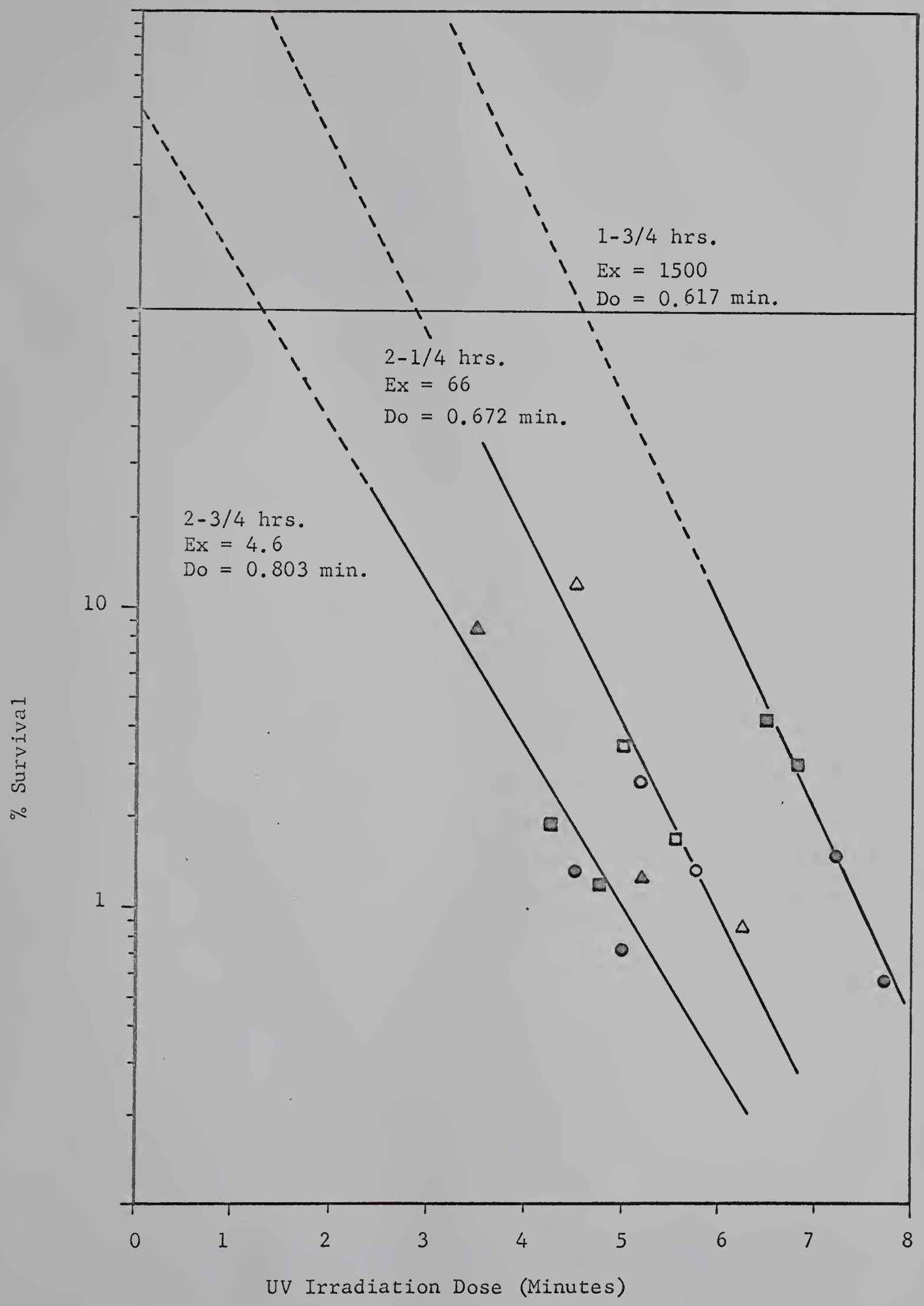
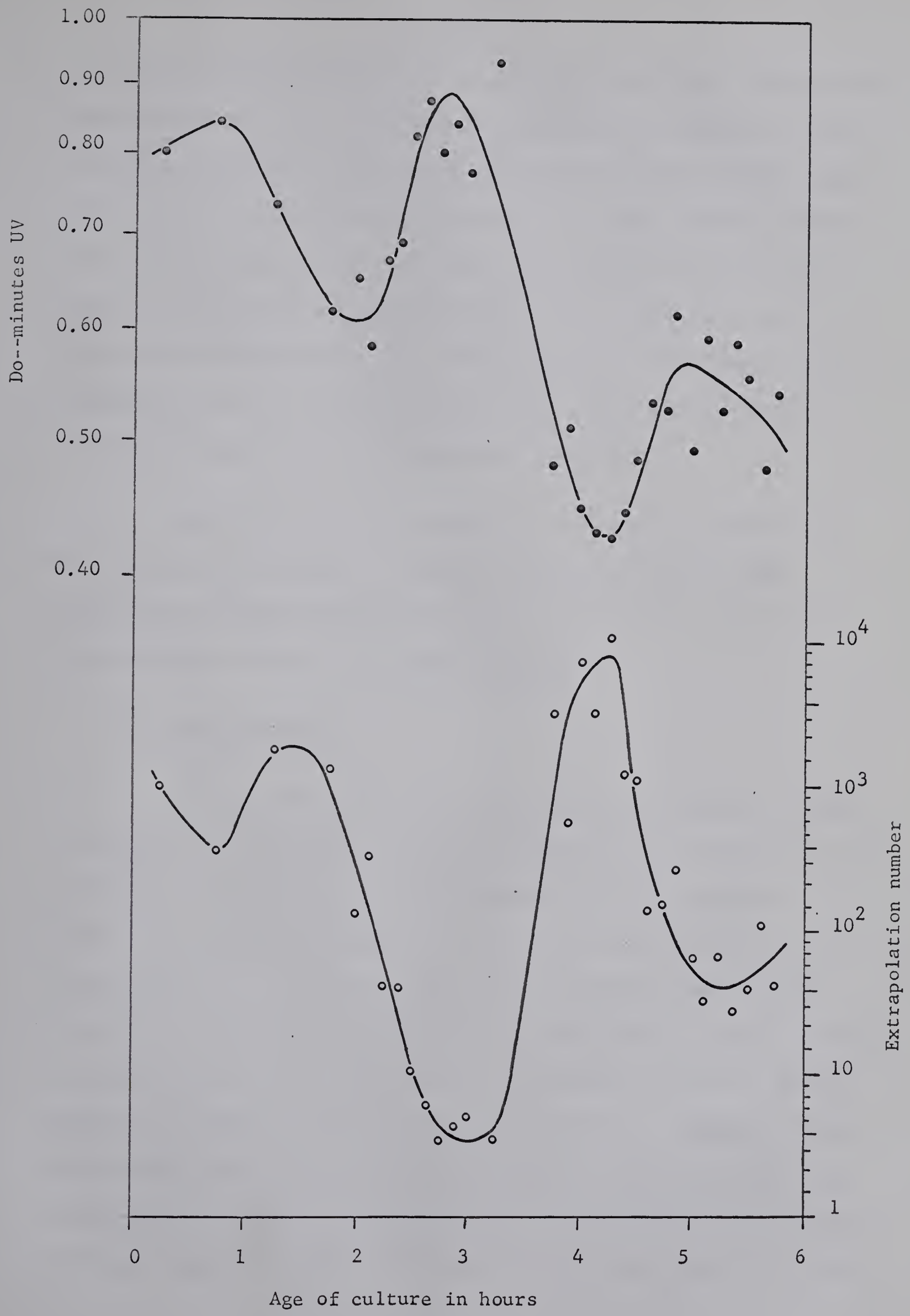

1-3/4 hrs.
Ex = 1500
Do = 0.617 min.
2-1/4 hrs.
Ex = 66
Do = 0.672 min.
2-3/4 hrs.
Ex = 4.6
Do = 0.803 min.
10
1
% Survival
0
1
2
3
4
5
6
7
8
UV Irradiation Dose (Minutes)

Fig. 6. Do-values (upper curve) and extrapolation numbers (lower curve) as a function of culture age. These curves are based on data obtained in five separate experiments (XIV to XVIII inclusive) in which cultures were synchronized by procedure A.


1.00
0.90
0.80
0.70
0.60
0.50
0.40
Do--minutes UV
10^4
10^3
10^2
10
1
Extrapolation number
0
1
2
3
4
5
6
Age of culture in hours

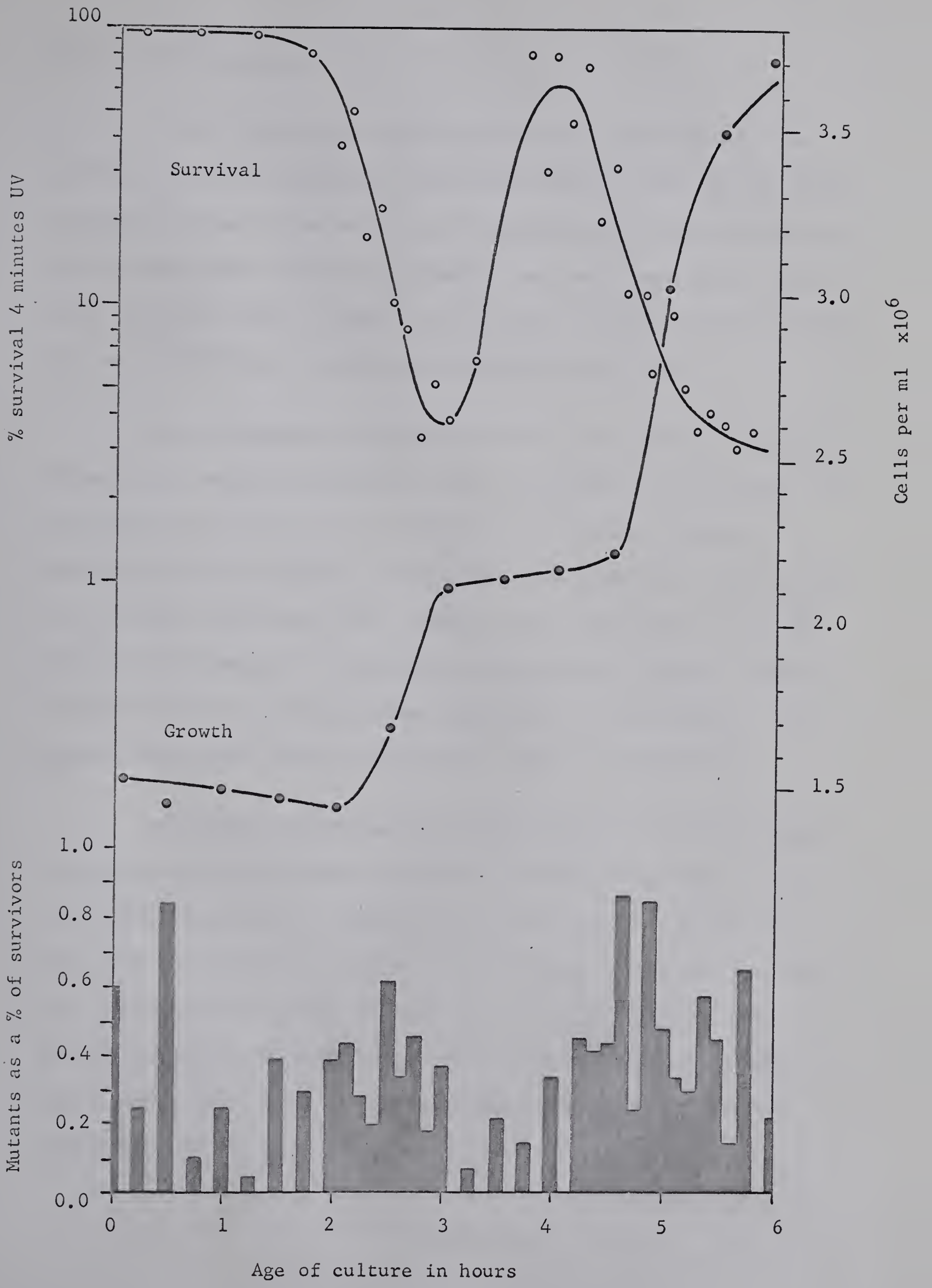
2-1/4 and 2-3/4 hours (Plate I). In the second cell cycle the sporidia begin budding at 3 hours and cell separation occurs between 4-1/4 and 4-3/4 hours (Plate 2). During the first cell division, nuclear separation occurs at 1-1/2 hours (Unrau 1965). Do values increase between 0 and 1 hour, then decline to a low of 0.58 minutes of UV at 2-1/4 hours. They increase to a high of 0.90 minutes of UV at 3 hours. The pattern is repeated in the second cell cycle. At 4 hours the Do values fall to a low of 0.43 minutes of UV. The pattern of change becomes less distinct as synchronization breaks down.

From Fig. 6 it can be seen that extrapolation numbers and Do values vary inversely. A similar relationship between these parameters was found by Whitmore et al.,(1965) in their study of X-ray sensitivity using L-cells grown in vitro.

(ii) Mutation rates

The rate of mutation for each culture-age treatment is shown in Appendix 8a. The number of mutants and surviving colonies that were analysed are totals obtained by summing the data of experiments IV to XVIII. A comparison of mutation rate to growth and UV sensitivity is shown in Fig. 7. Low mutation rates were obtained in the intervals from 3/4 to 1-1/4 hours and from 3-1/4 to 3-3/4 hours. It is difficult to relate both of these intervals of low mutation rate to any single feature of either the growth or the survival curves. However, if the data for the first two hours are not considered the low mutation rate occurs in the half-hour period following cell separation and is flanked on either side by periods of high mutation rate which occur at or just

Fig. 7. Survival (4 min UV at a rate of 630 ergs/mm^2/min), growth and mutation rate as a function of cell age. Survival is based on data obtained from experiments XIV to XVIII inclusive. Growth is based on the average of colony counts, for several sets of control plates (Appendix 2). Mutation rate is based on results obtained from experiments IV to XVIII inclusive, in which cultures were synchronized by procedure A. The UV dosage was adjusted for each culture age treatment in order to provide a surviving fraction ranging between 0.5% and 10%.


100
10
1
Survival
% survival 4 minutes UV
3.5
3.0
2.5
2.0
1.5
Cells per ml x10^6
Growth
1.0
0.8
0.6
0.4
0.2
0.0
Mutants as a % of survivors
0
1
2
3
4
5
6
Age of culture in hours

before cell separation.

Table I summarizes the data recorded in Appendix 8a. The mutation rates are expressed as percentages and are based on the number of mutant colonies recovered among those that have survived irradiation, for ten consecutive 5/8-hour intervals. Mutation rates ranged from a low of 0.11% to a high of 0.48%. The average of the fluctuating values over two cell-division cycles was calculated to be 0.32%.

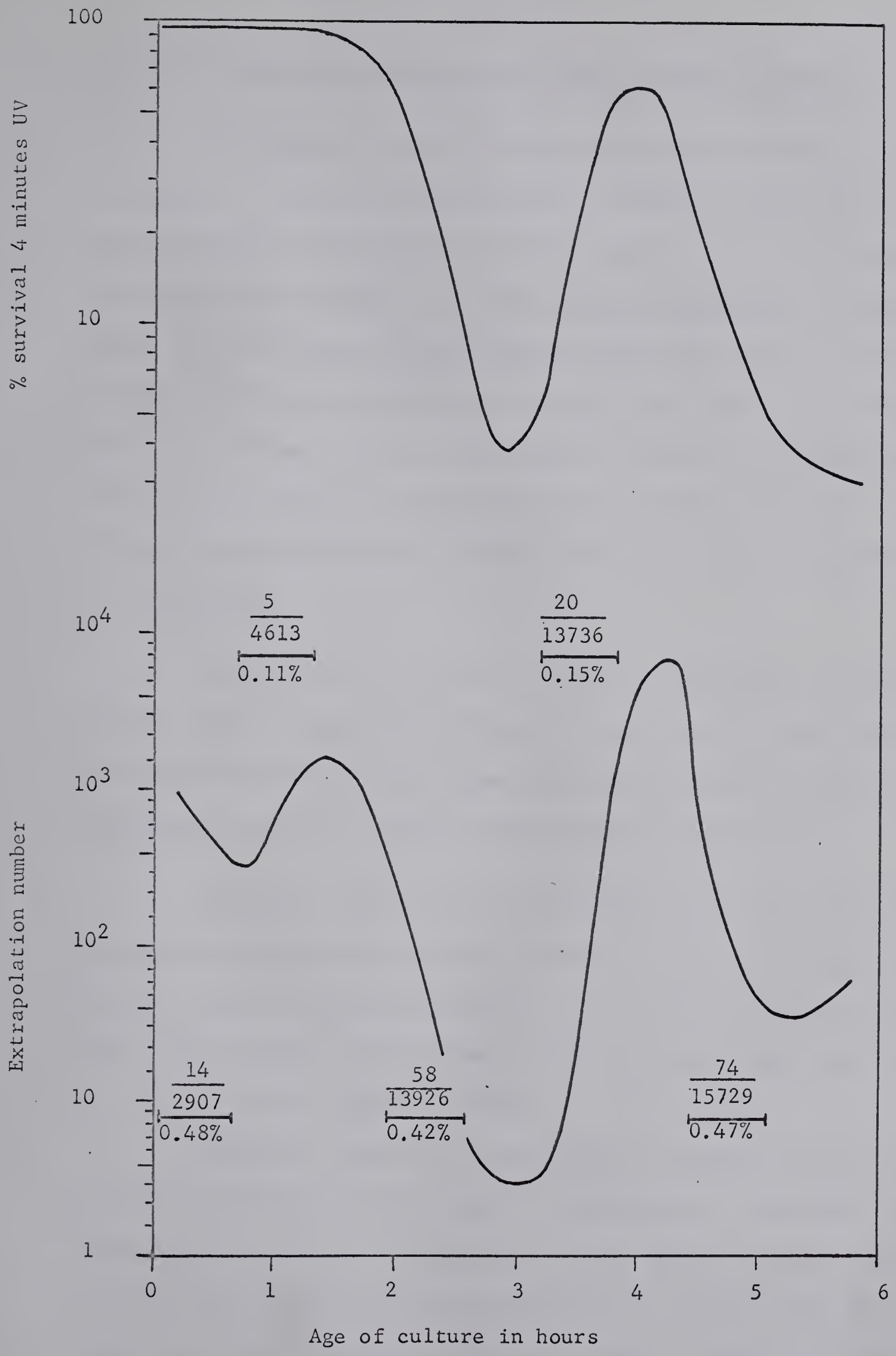
Fig. 8 compares the mutation rate to the changing pattern of extrapolation numbers through two cycles of synchronized division. As was pointed out earlier, it is difficult to relate the changes in mutation rate to the pattern of changing sensitivity to UV as represented by the upper survival curve. However, when one relates it to the extrapolation numbers, it is noted that low mutation rates are obtained in regions where the extrapolation numbers are on the increase. High mutation rates occur where extrapolation numbers are declining.

An analysis of mutation rates for different UV doses showed that there was no significant increase in mutation rate with increase in UV dosage for survival ranging between 0.5% and 8%. It was also found that for a particular mutant type there was no significant pattern of recovery throughout the cell cycle. An analysis of those mutants for which adequate numbers were obtained showed frequency changes whose pattern did not differ from that established for the overall mutation rate.

Table 1. Mutation rate as related to time following UV irradiation of a sporidial culture of U. hordei

Age of culture (hours)	Colonies surviving irradiation	Number of mutants obtained	Mutation as a % of survivors
0 to 3/4	2907	14	0.48
3/4 to 1-3/8	4613	5	0.11
1-3/8 to 2	5430	19	0.35
2 to 2-5/8	13926	58	0.42
2-5/8 to 3-1/4	13875	48	0.35
3-1/4 to 3-7/8	13736	20	0.15
3-7/8 to 4-1/2	6405	22	0.33
4-1/2 to 5-1/8	15729	74	0.47
5-1/8 to 5-3/4	16271	63	0.39
5-3/4 to 6-1/4	2508	10	0.40

Fig. 8. Survival (upper curve) and extrapolation numbers (lower curve) as a function of cell age. The percentage mutation rates for five culture-age intervals correspond to the respective regions of the curve for extrapolation numbers (synchronization procedure A). The UV dosage was adjusted for each culture age treatment in order to provide a surviving fraction ranging between 0.5% and 10%.


% survival 4 minutes UV
100
10
10^4
5
4613
0.11%
20
13736
0.15%
Extrapolation number
10^3
10^2
10
1
14
2907
0.48%
58
13926
0.42%
74
15729
0.47%
0
1
2
3
4
5
6
Age of culture in hours

(iii) Irradiation results using synchronization procedure B.

The combined results of experiments XIX and XX are presented in Appendix 7. These experiments were carried out using $I_4(+)$, as were all previous experiments (I to XIX incl.). The data on irradiation sensitivity of mutant V-441 are reported in Appendix 9 (experiment XXII). The Do values and extrapolation numbers for these two sets of experiments are plotted against culture age in Fig.9. It will be noted that although radiation responses (Do values and extrapolation numbers) as a function of culture age differ quantitatively from those obtained for experiments XIV to XVIII (See Fig. 6) they are qualitatively the same.

Mutation rate as related to survival and growth is shown in Fig. 10. The one significant feature is that mutation rates during the second cell cycle are much lower than that obtained for the corresponding position in cultures synchronized by procedure A.

Before the synchronization procedures, discussed above, were worked out a number of experiments had been carried out in which logarithmic cultures were irradiated with the objective of inducing mutations. The mutation rate that was observed in these early experiments was very low (in the range of 0.01%).

During the process of synchronization, changes occur in the sporidial growth-behavior which must be interpreted to mean that marked changes also occur in the physiology of these cells. If this is true then the extent to which the cell physiology is altered would be related to the time the sporidia are held in the so called stationary

Fig. 9. Do-values and extrapolation numbers as a function of culture age. Curves labelled "b" and "b^1" are based on experiments XIX and XX. Curves "a" and "a^1" are based on experiment XXII (synchronization procedure B). The UV dosage was adjusted for each culture age treatment in order to provide a surviving fraction ranging between 0.5% and 10%.

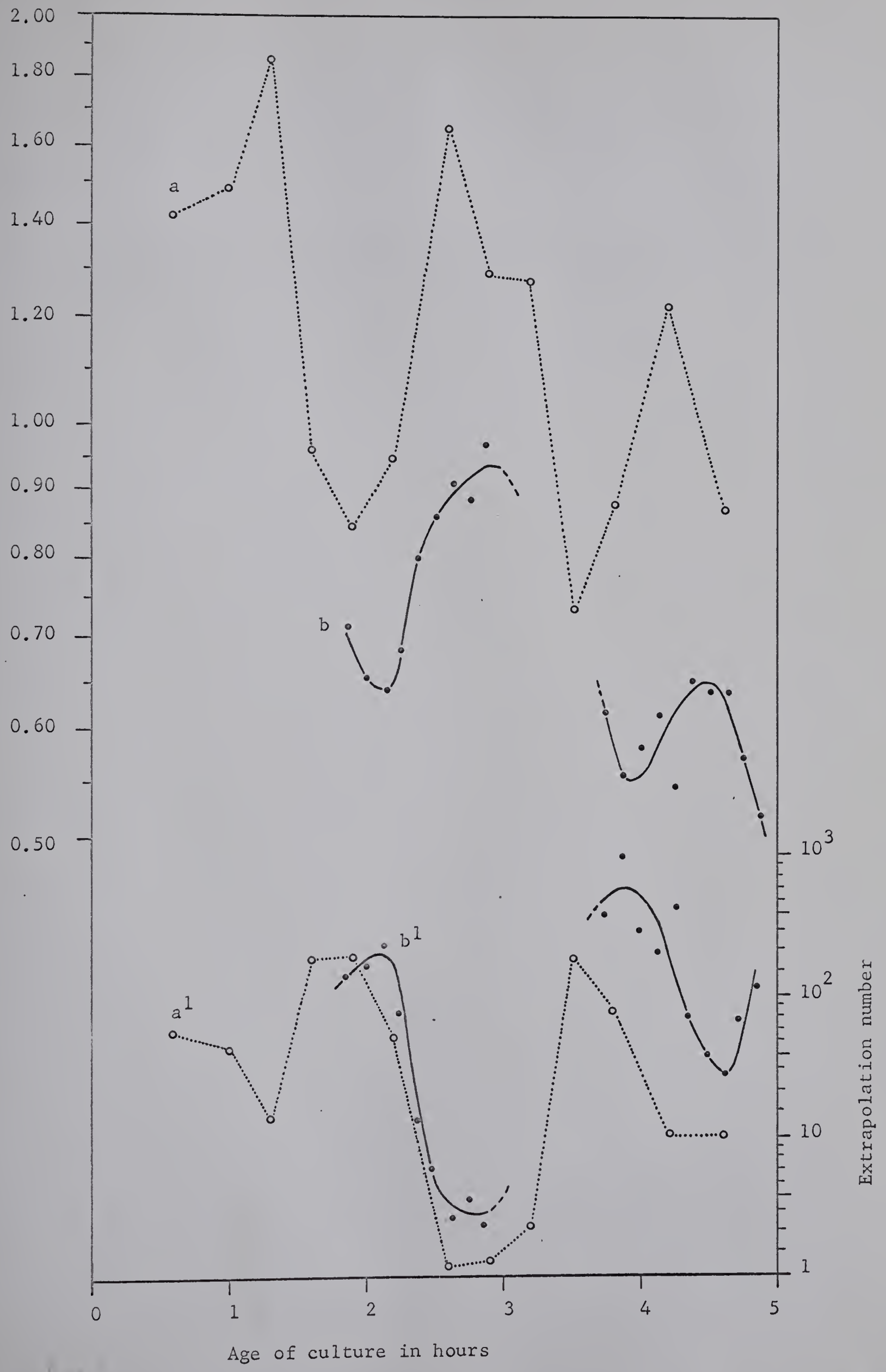
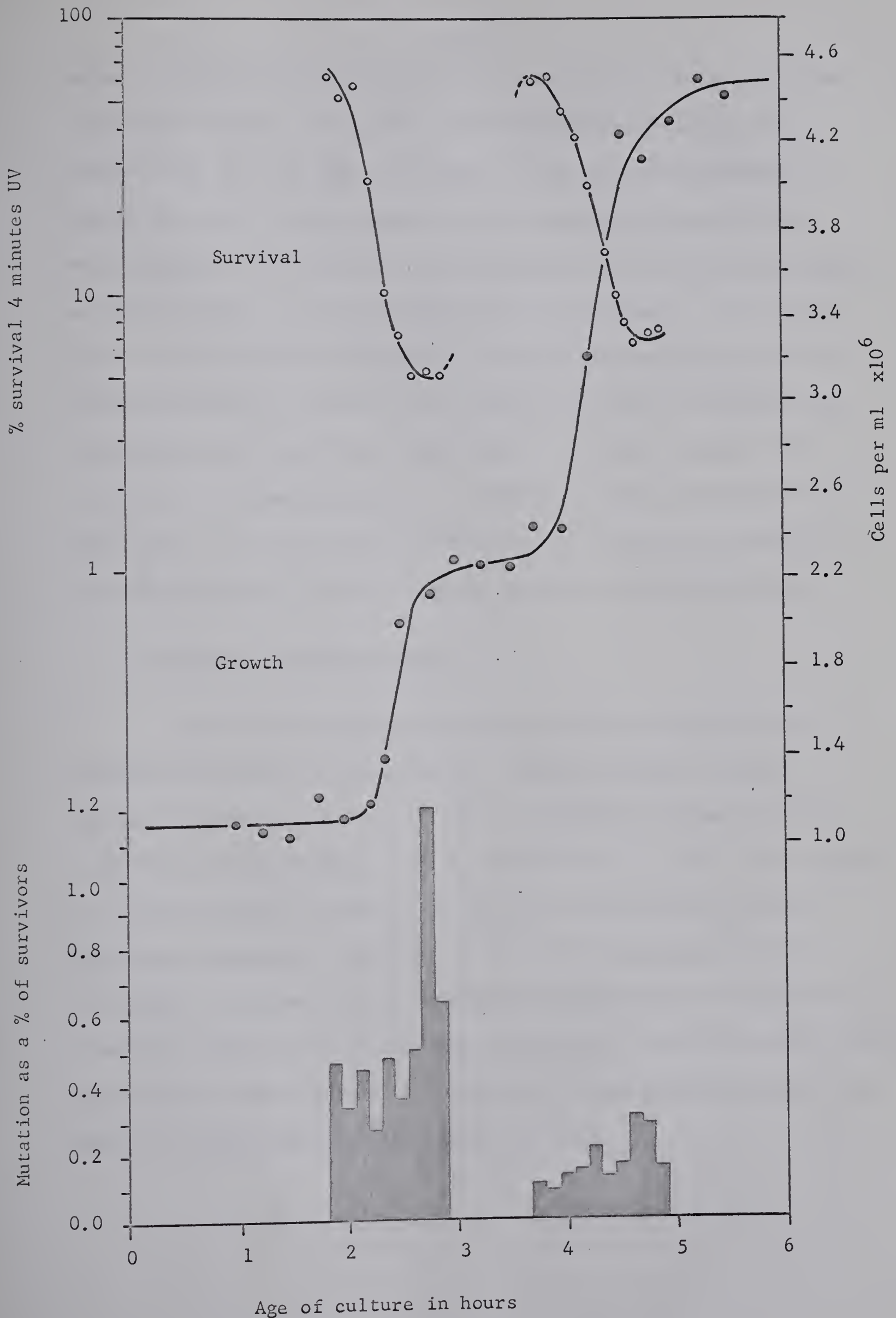

2.00
1.80
1.60
1.40
1.20
1.00
0.90
0.80
0.70
0.60
0.50
Do--minutes UV
a
b
a1
b1
0
1
2
3
4
5
Age of culture in hours
10^3
10^2
10
1
Extrapolation number

Fig. 10. Survival (4 min of UV), growth and mutation rate as a function of cell age for experiments XIX and XX. Synchronization procedure B was used in the experiments.

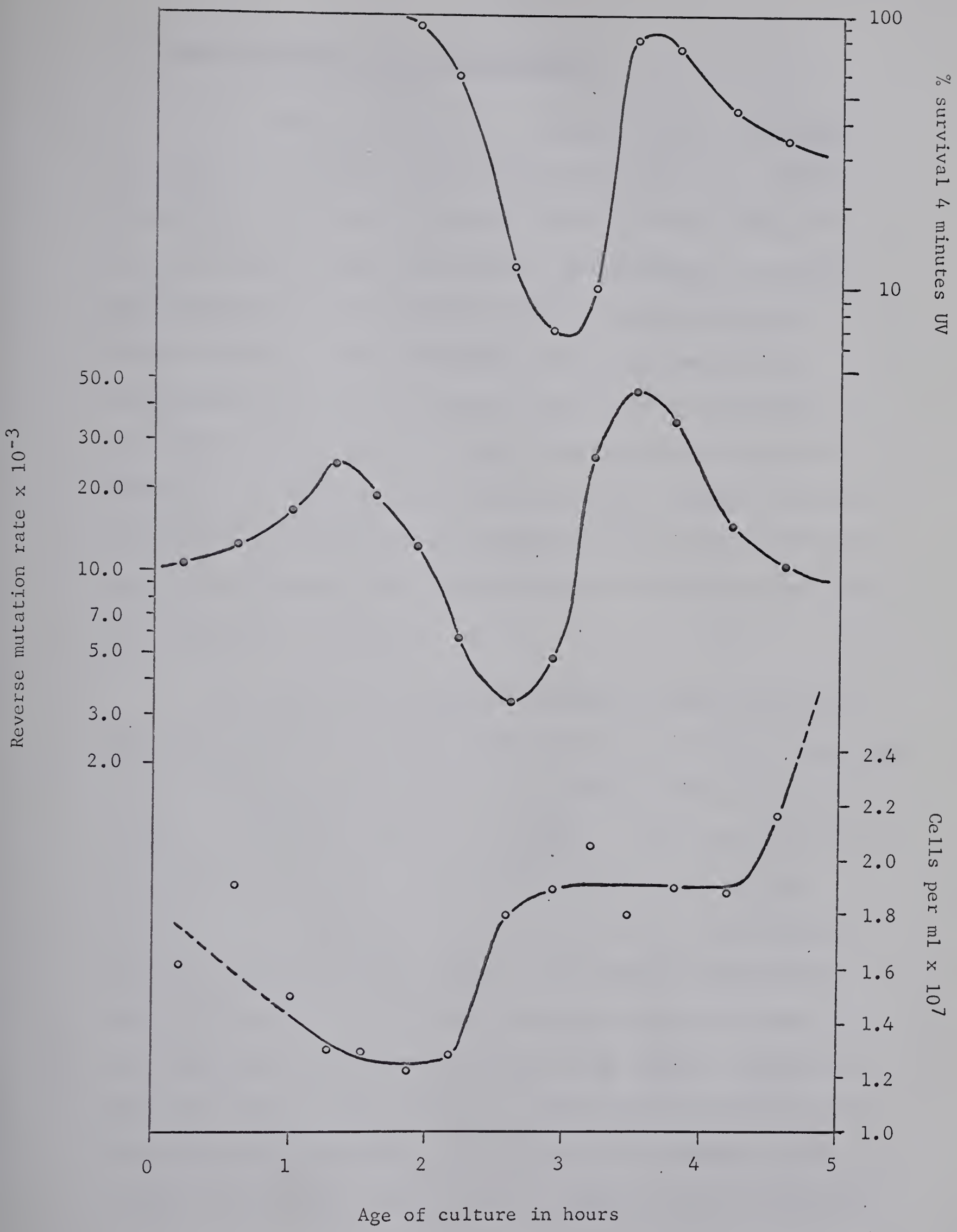

100
10
1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
% survival 4 minutes UV
Survival
Growth
Mutation as a % of survivors
4.6
4.2
3.8
3.4
3.0
2.6
2.2
1.8
1.4
1.0
Cells per ml x10^6
0
1
2
3
4
5
6
Age of culture in hours

phase. A comparison of synchronization procedures A and B indicates that type B cultures are held in the quasi-stationary phase for a shorter time than are type A cultures. With the reestablishment of growth one would therefore expect type B cultures to recover the physiological conditions that are characteristic of logarithmic growth more quickly than cultures synchronized by procedure A. If the mutation rates of irradiated logarithmic cultures are truly low then the low mutation rates observed in the second cell cycle of cultures synchronized by procedure B may well reflect an earlier recovery from the effects of synchronization. A corollary to this conclusion suggests that the overall high mutation rate is a function of changes in cell physiology that occur during the process of synchronization.

(iv) Reverse mutation studies

An unstable arginine mutant No. V-441 was irradiated and analysed for mutation to prototrophy. Reverse mutants occurred spontaneously at a rate of 7.5×10^{-4}. Irradiated samples showed reverse mutation rates ranging from 1.8×10^{-3} to 4.6×10^{-2}. The pattern of reverse mutation throughout two cycles of synchronized division showed two differences from that of the forward mutation rate: (1) the number of reverse mutants increased markedly with the increase in irradiation dose for all culture-age treatments, and (2) the peak reverse mutation rates were located at precisely the same positions as the low points of forward mutation rates (see Fig. 11).

Fig. 11. Survival (4 min of UV), reverse mutation rate and growth as a function of culture age for arginine mutant, V-441. Growth was based on colony counts taken from control plates. The UV dosage was adjusted for each culture age treatment in order to provide a surviving fraction ranging between 0.5% and 10%.


Reverse mutation rate x 10^{-3}
50.0
30.0
20.0
10.0
7.0
5.0
3.0
2.0
100
10
% survival 4 minutes UV
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
Cells per ml x 10^7
0
1
2
3
4
5
Age of culture in hours

4. Mutant Identification and Auxanography

All mutants produced in experiments IV to XX originated from the UV irradiation of the monosporidial line $I_4(+)$. These mutants were represented by the two series: (i) the V-series obtained in studies using type A cultures (experiments IV to XVIII) and (ii) the Z-series obtained in studies on type B cultures. Mutants produced in experimentation that preceded work on UV sensitivity of synchronized cultures (type A) were all derived from $E_3(-)$. Those obtained by X-ray irradiation were identified by means of a number preceded by the letter "X". Mutants obtained from $E_3(-)$ by UV irradiation were designated by the letter "U" and a number. All mutants were numbered consecutively in the order in which they were obtained.

An analysis of the mutants that were obtained from experiments IV to XVIII showed that certain mutant types were not represented or were not as frequent as might be expected according to the data presented by Holliday (1961) for U. maydis. These were mutants with deficiencies for niacin, thiamine, para-amino-benzoic acid and cytidine. All experiments up to this time had used a complete medium as specified by Vogel (1956). Since Holliday used notably higher concentrations of vitamins and nucleic acid hydrolysate, two experiments (XIX and XX) were performed using a Vogel's medium enriched with both of these nutrients. The recovery of niacin mutants was significantly increased (15 in a total of 157 mutants for Experiments XIX and XX, compared with 5 in 356 for all previous work),

though no mutants of the other three types were obtained. In this as in other organisms, the spectrum of mutants recovered was obviously influenced by the conditions of the experiment. (Wagner and Mitchell, 1964; Lein et al., 1948; Hans et al., 1952).

The classification of mutant types is given in Table 2. Large numbers of adenine, arginine and methionine mutants were recovered. This pattern of mutant recovery is similar to that reported for U. maydis (Holliday, 1961) and for other fungi discussed in the same paper (Penicillium by Bonner, 1946; Ophiostoma by Fries, 1947; Neurospora by Tatum et al., 1950; Aspergillus by Pontecorvo et al., 1953). The results of Experiments XIX and XX suggest that niacin mutants can be produced in large numbers in U. hordei as was reported for U. maydis. Further comparison with U. maydis shows two notable differences: (i) no cytidine, thiamine or para-amino-benzoic acid mutants were recovered in U. hordei, (ii) proline, lysine and the double-requiring isoleucine-valine mutants were recovered in U. hordei but not in U. maydis; proline mutants are commonly recovered in Penicillium (Bonner, 1946) and Neurospora (Srb, Fincham and Bonner, 1950); lysine mutants are commonly found in the four fungi mentioned above and isoleucine-valine mutants have been recovered in Neurospora (Bonner, 1943; Tatum et al., 1950).

All of the mutants in both the X- and the U-series, and most of those in the V-series were mated with normal (i.e. non-mutant) sporidia of the opposite sex. Teliospores were harvested from infected plants and inheritance studies were carried out on 82 of the different

Table 2. The types of nutritionally deficient mutants obtained following UV irradiation of prototrophic sporidia

Requirement	Genetic symbol	Numbers of mutants
Nucleic acid:		
Adenine	ad	66
Vitamins:		
Niacin	niac	20
Pantothenic acid	pan	17
Pyridoxin	pdx	9
Inositol	inos	14
Choline	cho	1
Amino acids:		
Arginine	arg	82
Proline	pro	25
Methionine	meth	66
Histidine	hist	42
Lysine	lys	47
Leucine	leuc	30
Isoleucine	iso	5
Isoleucine - Valine	iso-val	14
Serine	ser	18
Phenylalanine	phen	2
Undetermined		57
Total		515

Table 2a. Data recorded for the mating types of mutants segregated from the teliospores of 82 different heterozygous crosses.

Deficiency	Mutant no.	Mating type of segregants (-)	(+)	(0)	Deficiency	Mutant no.	Mating type of segregants (-)	(+)	(0)
Adenine	U-7	7	9		Proline	V-324	3	7	
	U-40	10	14			V-335	5	5	
	X-29	19	16			V-390	0	70	
	V-175	6	9			V-435	0	16	
	V-201	7	9						
	V-217	12	4		Methionine	U-25	9	6	
	V-288	5	5	3		V-55	3	9	
	V-443	5	11			V-158	4	5	
						V-166	5	6	1
Pantothenic	V-52	10	6			V-241	7	5	
acid	V-191	0	16			V-274	6	6	
	V-227	0	58			V-319	1	4	5
	V-271	8	8			V-336	8	4	
	V-292	11	5			V-364	3	9	
	V-328	0	79			V-370	7	5	
	V-346	4	6						
	V-359	5	5		Histidine	V-111	6	5	
	V-445	0	70			V-382	6	8	2
						V-426	7	3	3
Niacin	U-29	5	11			V-466	5	10	1
	X-100	8	7			V-469	9	5	
Pyridonine	V-26	5	4		Lysine	V-93	6	10	
	V-203	4	3			V-147	6	7	
	V-210	3	5			V-329	11	3	2
	V-299	4	6			V-345	8	7	
	V-388	4	5			V-433	6	9	1
Inositol	V-196	5	5		Leucine	U-38	11	15	
	V-198	4	5			V-343	10	5	
	V-256	6	4			V-378	5	8	3
	V-406	5	5			V-394	11	5	
						V-407	3	11	
Choline	V-338	5	10			V-417	5	5	
						V-419	3	2	11
Arginine	X-52	12	5						
	V-240	3	7		Isoleucine	V-13	8	7	
	V-242	5	5			V-174	4	12	
	V-244	9	7						
	V-261	9	7		Iso-leucine-	U-26	12	4	
	V-267	6	10		valine	U-50	7	8	
	V-293	7	9			V-377	11	6	
	V-376	7	3			V-449	10	6	
	V-393	5	5			V-453	11	4	
	V-400	6	4						
	V-423	5	11		Serine	V-5	7	9	
	V-436	5	5			V-138	3	13	
						V-451	10	6	

mutant-by-normal crosses. Data relating to linkage relationships between the mutant locus and the mating-type locus are recorded in Table 2b. Individual mutants of two types of deficiency, pantothenic acid and proline, are apparently closely linked with the mating locus. Other mutants of both types appeared to segregate independently of the mating locus. As will be seen later, none of the other mutant types, with the possible exception of the arginine/ornithine group, showed evidence of linkage with the mating locus.

(i) Nucleic Acid Mutants

Only mutants showing a requirement for adenine were obtained. Although most of these also responded equally well to hypoxanthine a few did not grow, or grew only poorly on this nutrient (V-25, V-85, V-201, V-215b*, V-288a*, V-295b*); many of these produced a red pigmentation after several days of growth on adenine.

A peculiarity noted in the adenine mutants was that after a period of storage several segregant types appeared in the cultures. Most of these variants showed only differences in morphology, especially with regard to color differences (green, black, brown and red). Segregants from a few mutant strains, however, showed distinct differences in their nutritional requirements. Mating studies have not yet been carried out to establish the genetic integrity of these segregants.

* Small letters following the mutant number refers to a segregant obtained from the original mutant.

(ii) Vitamin Mutants

Thirteen auxotrophs that respond to niacin and to intermediates in the metabolic pathway leading to niacin are reported in Table 3. The niacin mutants fall into three categories: (i) those that respond only to niacin; (ii) those that respond to anthranilic acid, 3-hydroxy-anthranilic acid and to phenylalinine in addition to niacin and (iii) those that respond to L-tryptophane and to L-3-hydroxykynurenine in addition to the chemicals of the above groups. Only mutants with the single niacin requirement (group i) do not respond to phenylalanine. The mutants recorded in Table 3 do not respond to shikimic acid or to L-kynurenine sulfate. These data suggest that there are two metabolic pathways leading to niacin, only one of which involves tryptophane. Two phenylalanine mutants are reported in Table 3 because of their natural affinity to this group.

The single choline mutant (V-338) grew on N-methylethanolamine and choline, but not on ethanolamine-HCl or dimethylethanolamine-HCl. This is surprising since dimethylethanolamine is expected to be an intermediate between monomethylethanolamine and choline. Both pantothenic acid and pyridoxine mutants showed vigorous growth when their respective nutrient requirements were provided at a rate of 1 mg/litre (see Methods and Materials). The growth of inositol mutants was improved when the inositol supplement was increased to 5 mg/litre.

On the basis of segregation studies the pantothenic acid mutants were found to be of two types: (i) those that were closely linked to the mating locus (V-191, V-227, V-328 and V-445) and

Table 3. Data on auxanographic studies with 13 niacin and two phenylalanine mutants

Mutant no.	Response** to: L-phenyl-alanine	L-trypto-phane	L-3-hydroxy kynurenine	Anthranilic acid	3-hydroxy anthranilic acid	Niacin
Z-3*	-	-	-	-	-	5
Z-10*	-	-	-	-	-	5
Z-21*	-	-	-	-	-	5
Z-112*	-	-	-	-	-	5
Z-203*	-	-	-	-	-	5
X-100	3	-	-	3	3	3
Z-110*	5	-	-	5	5	5
Z-142*	5	-	-	5	5	5
Z-198*	5	-	-	5	5	5
U-29	5	4	5	5	5	5
Z-53*	5	4	5	5	5	5
Z-55*	5	5	5	5	5	5
Z-13*	5	2	5	5	5	5
V-403*	5	-	-	-	-	-
Z-7*	5	-	-	-	-	-

Mutants in the group were also tested on shikimic acid and L-kynurenine sulfate; responses were all negative.
Mutants that responded to phenalanine, also grew equally well on sodium phenylpyruvate.

* Mutants mated but no teliospores available.
** Response to supplement is graded in arbitrary units from 1 to 5;
- indicates no response; 5 represents normal growth.

(ii) those that appeared to segregate independently of it (V-52, V-271, V-292, V-346 and V-359). Assuming that type (i) mutants all belong to a single locus then this locus must be located on the same chromosome as the mating locus and closely linked to it. If it is further assumed that mutants of the second type also belong to a single locus, then at least two pantothenic acid loci are needed to explain the data. Since the haploid chromosome number most commonly reported for species of Ustilago is two (Kharbush, 1927; Wang, 1934; Person and Wighton, unpublished), the second locus must be located either on the other chromosome or on the same chromosome as the mating locus and at some distance from it.

(iii) Amino Acid Mutants

Arginine and proline. Table 4 shows the responses of 22 mutants to L-glutamic acid, L-proline, L-orthinine, L-citrulline and L-arginine. Proline and arginine mutants are known to be associated in the same metabolic pathway in Neurospora (Srb, Fincham and Bonner, 1950) and in Salmonella typhimurium (Miyake and Demerec, 1960). This association is also apparent for proline and arginine mutants of U. hordei (See V-324, Table 4). Of the mutants which respond to arginine, two groups are evident: (i) those that respond to arginine alone; and (ii) those that respond to ornithine and citrulline in addition to arginine.

Two proline mutants (V-390, V-435) showed close linkage to the mating locus while two other proline mutants (V-324, V-335) recombine freely with the mating locus (see Appendix II). On the basis

Table 4. Results from auxanographic studies on 7 proline and 15 arginine mutants

Mutant no.	Response*** to: Minimal	L-glutamic acid	L-proline	L-ornithine	L-citrulline	L-arginine
V-95*	-	-	5	-	-	-
V-324	-	-	5	5	5	-
V-335	-	-	5	-	-	-
V-379*	-	-	5	-	-	-
V-390	-	-	5	-	-	-
V-435	-	-	5	-	-	-
Z-167*	-	-	5	-	-	-
V-240	-	-	-	5	5	5
V-293	-	1	1	5	5	5
V-423	-	-	-	5	5	5
V-436	-	-	-	5	5	5
U-51**	-	-	-	5	5	5
X-52	-	-	-	5	5	5
V-242	-	-	-	1	-	5
V-244	-	-	-	1	-	5
V-261	-	-	-	1	1	5
V-267	-	-	-	2	1	5
V-393	-	-	-	1	-	5
V-400	-	-	-	2	1	5
U-52**	-	-	-	2	1	5
X-58	-	-	-	2	1	5
Z-205*	-	-	-	-	-	5

* Mutants mated but no teliospores available.
** Teliospores available but no segregation performed.
*** Response to supplement is graded in arbitrary units from 1 to 5;
- indicates no response; 5 represents normal growth.

of the small number of segregants analysed it is impossible to decide if the recombination in these last two mutants is random with respect to the mating-type locus. A consideration of the arginine/ornithine mutants suggests that these also may be linked to the mating locus. Summing the ratios for these [X-52 mated as (-); V-240, V-293 and V-436 mated as (+)] showed that non-recombinants outnumber recombinants by 44 to 25, a ratio that yields a Chi-square value significant at the 2% level. Data for larger numbers of segregants will be needed before a definite conclusion can be drawn with respect to these possible linkage relationships. However, if it should be established that these mutants, along with proline mutants of the type represented by V-324 and V-335, are all linked, then one may have a metabolic sequence represented by a series of genes linearly arranged with proline at one end closely linked to the mating locus and arginine segregating freely at the other end. Several cases of an assembly line of linked loci have been described: for histidine (Hartman et al., 1960), for proline (Miyake and Demerec, 1959) and for threonine and isoleucine-valine (Glanville and Demerec, 1960).

Methionine. The responses of 15 methionine mutants to sodium thiosulfate, L-cysteine, L-homoserine, DL-cystathionine, L-homocysteine and L-methionine are shown in Table 5. The requirements in these mutants appear to be the same as those reported for methionine mutants in Neurospora (Wagner and Mitchell, 1964).

Leucine, isoleucine and valine. Table 6 shows data on 16 mutants which are representative of this group. Out of a total of

Table 5. Results of auxanographic studies on 15 methionine mutants

Mutant no.	Response** to: Minimal	Na Thiosulfate	L-cysteine	L-homoserine	DL-cystathione	L-homocysteine	L-methionine
V-241	-	5	5	-	-	3	3
V-311*	-	5	5	-	-	1	3
V-158	1	2	5	1	3	4	1
V-364	-	1	5	-	-	5	5
Z-108*	-	-	5	-	-	5	5
Z-179*	-	-	5	-	-	5	5
V-200*	1	2	-	5	2	4	5
V-274	-	-	-	-	5	2	5
V-319	-	-	-	-	5	2	5
V-336	-	-	-	-	5	2	5
U-25	-	-	-	2	-	4	5
V-55	-	-	-	1	-	2	5
V-91*	-	-	-	1	-	1	5
V-370	1	-	-	1	-	2	5
Z-175*	-	-	-	-	-	-	5

* Mutants mated but no teliospores available.
** Response to supplement is graded in arbitrary units from 1 to 5; - indicates no response; 5 represents normal growth.

49 mutants recovered in the group,30 showed a requirement for leucine, 5 for isoleucine and 14 for both isoleucine and valine together. Mutants with double requirements for isoleucine and valine have been studied extensively in Neurospora (Bonner et al., 1943; Bonner, 1946a; Bonner, 1946b; Kiritani, 1962) and in Salmonella (Wagner and Bergquist, 1960). It was found that optimum growth was obtained with nutrient ratios of 70% valine and 30% isoleucine and that this growth could be further stimulated by the addition of leucine. Leucine was found to have a similar stimulating effect on U. hordei mutants. No studies were carried out using varied ratios of valine and isoleucine.

Mutant segregation by random plating showed that recovery ratios for these double requiring valine-isoleucine mutants were very low. This probably reflects the slow growth rate of these mutants. In addition to this observation, mutant colonies on complete plates were found to be much smaller than the prototrophs. The low segregation ratios might be improved by attempting to reduce the differential in growth rate that exists between mutant and prototroph colonies. This may be partially accomplished by using a specific medium, containing controlled amounts of valine, isoleucine and leucine.

Histidine. Segregation within mutant cultures, similar to that described for the adenine mutants, was also observed in the histidine mutants. Most of the variants differed in color; a few showed variations in nutritional requirements. Mutants in the histidine groups were of two types: some responded to histidine alone (V-111, V-382, V-466) whereas other responded to both histidine and histidinol

Table 6. Results of auxanographic studies on nutritional mutants of Ustilago hordei which show deficiencies for leucine, isoleucine and isoleucine-valine

Mutant no.	Response** to: Minimal	Leucine	Isoleucine	Valine	Isoleucine valine	Alpha-keto iso-caproic acid
U-38	-	5	-	-	-	5
V-343	-	5	-	-	-	5
V-378	-	5	-	-	-	5
V-394	-	5	-	-	-	5
V-407	-	5	-	-	-	5
V-417	-	5	-	-	-	5
V-419	-	5	-	-	-	5
V-13	1	1	5	-	5	-
V-174	1	1	5	-	5	-
Z-64*	-	-	5	-	5	-
Z-147*	-	-	5	-	5	-
V-377	-	-	-	-	4	-
V-449	-	-	-	-	4	-
V-453	-	-	-	-	4	-
U-26	-	-	-	-	3	-
U-50	-	-	-	-	3	-

Mutants in this group were also tested on alpha-keto-isovaleric acid; responses were all negative.

* Mutants mated but no teliospores available.

** Response to supplement is graded in arbitrary units from 1 to 5; - indicates no response; 5 represents normal growth.

(V-426 and V-469). Many mutants in the group grew slowly on their respective supplements. In addition to the reduced growth rate, and probably as a consequence of it, the rate of recovery of the mutant among random isolates from teliospores was low.

Lysine. Out of the 47 lysine mutants recovered, four responded to alpha-amino-adipic acid (147c, Z-122, Z-162, Z-178). This suggests that the biosynthetic pathway for lysine in U. hordei is similar to that reported for yeasts, Neurospora, protozoa and plant viruses (Good et al., 1950; Gilvarg, 1960; Sagisaka and Shimura, 1960). Mutant recovery by random isolation was generally low. Compared with the normal, mutants in this group have noticeably slower growth rates.

Serine. Mutants of this group also respond equally well to glycine. They have slower than normal growth rates and using random isolation techniques, mutant recovery is low.

Phenylalanine. Two phenylalanine mutants were recovered (see Table 3). As yet no host infections have been produced with these two mutants and no teliospores are available for study.

(iv) Undetermined Mutants

Most of the mutants in this group grew well on yeast extract. Only three required a complete medium for growth. A large number of them grew on a combination of vitamins and amino acids but did not grow on either of the nutrient mixtures separately. Mutant V-90 grew on nucleic acid hydrolysate, though it did not show a response to any of the individual nucleic acids. Possibly this mutant contains

a block preventing nucleoside or nucleotide formation.

(v) General Considerations

Bauch tests were carried out on a minimal medium in which the dextrose content was reduced to 2-3 g per litre. Certain of the mutant types studied gave very poor Bauch tests on this medium. (Table 2a: Ad. V-288; Meth. V-166 and V-319; Hist. V-382, V-426 and V-466; Lys. V-329 and V-433; Leuc. V-378 and V-419). Possibly a further reduction or complete elimination of the dextrose would give better results.

During the segregation studies it was noted that excessive concentrations of teliospores in the germinating medium tended to cause mycelial development and mating of adjacent promycelial cells before sporidia were produced. In order to ensure good sporidial segregation the spore concentrations should be kept at about 10^5/ml.

5. Mutant Virulence

There appeared to be little or no reduction in virulence when mutant/normal compatibility types were used to infect the susceptible varieties, Hannchen and Vantage. Similar observations were noted when two different mutants were used together to infect these varieties.

From the selfing of 11 mutants only two produced teliospores; one of these, U-38, must be considered doubtful. When crosses were made between different mutants showing the same nutritional requirements infectivity occurred in 7 out of 9 crosses. Two crosses ad U-7(+)/ ad U-19(-) and ad U-7(+)/ad X-137(-) did not produce teliospores (Table 7).

Table 7. Infectivity obtained from matings between mutants: (a) selfed, (b) differing in origin but showing similar nutritional requirements.

	Mutant		Mating			Infectivity	Approximate no. of plants
(a)	ad	(U-7)	selfed			nil	240
	ad	(U-40)	selfed			nil	80
	ad	(X-29)	selfed			nil	400
	ad	(X-137)	selfed			nil	80
	meth	(U-25)	selfed			nil	240
	leuc	(U-38)	selfed			1 plant	240
	ser	(U-43)	selfed			nil	240
	arg/orn	(X-52)	selfed			nil	80
	niac	(U-29)	selfed			some	240
	niac	(X-100)	selfed			nil	80
	hist	(X-1)	selfed			nil	240
(b)	ad	X-29 (+)	ad	U-7	(-)	light	80
	ad	U-7 (+)	ad	X-29	(-)	light	80
	ad	U-7 (+)	ad	U-40	(-)	light (nil on Hannchen)	80
	ad	U-7 (+)	ad	U-19	(-)	nil	80
	ad	U-7 (+)	ad	X-137	(-)	nil	80
	meth	U-25 (+)	meth	U-32	(-)	heavy	80
	meth	U-25 (+)	meth	U-47	(-)	heavy	80
	niac	U-29 (+)	niac	X-100	(-)	heavy	80
	arg	V-1 (+)	arg/orn	X-52	(-)	moderate	80

DISCUSSION

As was mentioned in the introduction an initial objective in this study was to produce a number of biochemically-deficient mutants of U. hordei in the expectation that these would prove useful in investigating the nature of host-parasite interactions. Although more than 500 mutations were induced it was possible to study the parasitic behavior of only a few genotypic combinations. In these preliminary studies it was found that parasitic cultures heterozygous for one and in some cases two mutant genes were generally capable of inducing the disease in susceptible varieties. From the somewhat limited information that was obtained it was also found that no disease was produced on at least two susceptible hosts, Hannchen and Vantage, when the infective culture were homozygous for a particular biochemical deficiency (with the possible exception of niacin mutants). This latter observation suggests that the nutritional requirements of the parasite are not freely supplied even by a susceptible host variety. With extensive testing it may be found that a mutant when selfed is capable of specifically infecting a certain host variety. If such a discovery were made it would establish a definite relationship between pathogen development and a biochemical pathway in the host. It would then be of considerable interest to carry out quantitative analyses on the synthetic activity of this pathway in both resistant and susceptible host varieties. To establish the biochemical basis of a gene for gene interaction in a host-parasite relationship would be, indeed, a most exciting discovery.

A study of nutritional mutants from the standpoint of their growth on the host suggests some features that might be comparable to certain aspects of mutants in other organisms. If a homozygous infective culture were to specifically infest a certain host variety it could be regarded as a type of conditional mutant not at all unlike the temperature-sensitive mutants, which are found in other organisms, and the amber mutants, which have been discovered in the T-even phages. The evidence available from mutant infectivity of the host indicates, that while selfed mutants are generally non-infective, crosses between two different mutants showing the same biochemical deficiency are often quite successful in producing an infection. This phenomenon suggests that the two different mutants may be capable of complementing one another with the result that together they enable the parasite to successfuly complete its life cycle in the host. It would be most interesting to find that the degree of infection was, to a certain extent, a measure of the ability of two mutants to compensate for each other's deficiency. If an infection were not produced by a cross between two different mutants that showed the same type of deficiency, it would suggest that the two mutants were affecting the same functional region of the genetic material.

Preliminary studies on the several types of nutritional mutants of U. hordei indicate that the biosynthetic pathways in this organism are similar to those established in other organisms. Since this organism is parasitic during a portion of its life cycle (Appendix I) these mutants should prove to be valuable in exploring the nature of the host-parasite relationship.

The non-parasitic haplo-phase is saphrophytic and quite able to grow on a very restricted substrate, containing inorganic salts and a simple carbon source. The parasitic phase which is initiated following dikaryotization is obligate, depending entirely upon its association with the host. In exploring the nature of the host-parasite relationship the investigator is continually confronted by two intriguing problems: the first which was discussed above involves the nature of the host-parasite interaction; the second involves the nature of the change that occurs in the metabolic pattern following dikaryotization.

The signal that brings about recognition of opposite mating types has been shown to involve a volatile chemical substance (Nielsen 1965). It is uncertain whether a similar substance (though not identical) is produced by both sex-type alleles. It is possible that the mating-type locus, besides controlling the attraction of opposite mating types to one another, might also control the metabolic changeover that occurs following dikaryotization. The use of mutants may be of assistance in studying the possibility of this type of action in the mating-type locus. The close association of certain pantothenic acid and proline mutants with the mating type locus may be of value in carrying out these studies. In addition to being useful as genetic markers, their close association with the mating-type locus may be of assistance in investigating the chemical substance that may be associated with this locus.

Another possibly significant relationship between different mutations and the mating-type locus was indicated by the relative vigor with which the different mutant-type segregants reacted with the two

opposite mating-type reference cultures used in carrying out the Bauch test. The segregants obtained by germinating teliospores of some mutant types, especially those with proline and pantothenic acid deficiencies, showed vigorous Bauch-test reactions. Segregants of other mutant types such as those with leucine deficiencies gave an extremely variable response to the Bauch test. It was impossible to make a positive sex-type determination on many of the segregants of particular mutants of this latter type(i.e. those of V-419, Table 2a). More extensive studies of these Bauch-test reactions using different mutant types in varied combinations with one another, on variably supplemented minimal medium might provide information that will be very useful in interpreting the nature of the mating reaction.

Inheritance studies have indicated that certain pantothenic acid and proline mutants were closely linked to the mating-type locus. Some of both types of mutants, however, do segregate independently of the mating-type locus. If a single gene were involved then chromosomal aberrations, such as inversions and translocations, might be implicated as causing the varied linkage relationship that exists between these two types of mutants and the mating type locus. At least one of these groups of mutants, either those that are closely linked with, or those that segregate independently of the mating type locus might consist of mutants that arise as a result of changes in position of a chromosomal segment. It would seem improbable that either group of mutant can be explained by the occurrence of two events, a mutation plus either an inversion or a translocation. Since pantothenic acid and proline

mutants are about equally divided into two categories on the basis of linkage with the mating-type locus, and since these two types of mutants appear to be the only ones showing a dual linkage relationship with the mating-type locus it would seem unlikely that either inversion or translocations are implicated in the linkage differences that exist between different pantothenic acid and proline mutants and the mating-type locus.

A simpler explanation is to assume that at least two genes are operating in each of the biosynthetic pathways leading to the synthesis of proline and pantothenic acid and that one of the genes in each pathway is located close to the mating-type locus. A close linkage relationship between these two mutant loci and the mating-type locus raises a question as to the nature of the mating type locus: is the mating-type locus an actively synthesizing region (gene) in its own right or is it a composite made up of several independent genes which are very closely linked to one another. Certainly, the mutant condition in which deficiencies exist for either pantothenic acid or proline, does not indicate any alteration of mating behavior as measured by the Bauch test when mutant cultures are combined with a normal culture of the opposite mating type.

The close linkage of certain mutants of both proline and pantothenic acid to the mating-type locus suggests that these two types of mutants are themselves closely linked to one another. It would be difficult to conceive of three independent loci, linearly arranged in one chromosomal strand that would not yield recombinants

when adequate numbers of mutant segregants are analysed. More extensive work should yield recombinants between the mating-type locus and either or both of the two mutant types. When recombinants are obtained the linkage relationships between the two mutants as well as the linkage between either mutant and the mating locus would be subject to more rapid analyses. Any prototrophs that arise from the cross pro(+), mating type (+), pan (-) by pro(-), mating-type (-), pan (+) would be recombinant types.

The second objective of this work was to study the responses obtained in synchronously-dividing cultures following UV-irradiation treatment. Although the responses recorded were similar to those obtained with other organisms following X-ray irradiation, this is the first comprehensive study of responses to UV irradiation in synchronously dividing cultures. Because of the similarity of the results obtained in this study to those of earlier studies the methods used earlier in analysing the responses obtained with human cell populations following X-ray treatment (Whitmore et al, 1965; Terasima and Tolmach, 1963) have been followed. This analytical approach has its basis in the "target" and "hit" theories and the question may therefore be raised whether it can be legitimately applied to the results of UV studies.

A main difficulty lies in accounting for the large extrapolation numbers that were obtained. These are not peculiar to UV-irradiation survival curves. Moseley (1963, cited in Oliver et al. 1964) obtained extrapolation numbers of 5000 to 10,000 from X-ray irradiation survival curves of Micrococcus radiodurans. When extra-

polation numbers of this magnitude are obtained, doubt is raised as to the applicability of the target theory. It is difficult to reconcile extrapolation numbers of 5,000 to 10,000 with the inactivation of 5,000 to 10,000 individual targets as would be required of a strictly multi-target hypothesis. It was shown by Hall, Oliver and Lajtha (1962) that the inactivation of two or three targets could lead to a logarithmic survival curve with an apparent extrapolation number of 6, and that this was consistent with the multi-target theory. On the other hand, it was shown by Oliver et al. (1964) that the multi-hit theory was also applicable. Extrapolation numbers of the magnitude obtained by Moseley could be accounted for by some 10 hits in a single larger target having only about twice the volume of that corresponding to each of the 10,000 individual targets of the former theory.

The mathematical applications of Oliver et al. (1964) and of Fowler (1964) have shown that the multi-hit single-target model, though postulating survival curves which have increasing degrees of slope at higher irradiation doses, does not require survival curves which approach the extent of curvature obtained by Barendsen et al. (1960). They further point out that curves of the multi-hit model can be applied to most experimental data, and that experimental errors present in this type of work make it difficult to distinguish conclusively which model provides the best fit.

Assuming that the multi-hit model applies to the data obtained on survival curves in irradiation studies of U. hordei

one is faced with the problem of assessing the validity of the extrapolation numbers for this model. Extrapolation numbers obtained from survival curves of this model have been said to have no meaning. This statement is made on the basis of two objections: one, that no numerical target can be assumed to correspond with the extrapolation number; and two, that the curvature of the survival curves increases with decreasing survival. In the light of the above discussion neither objection seems to apply as long as no attempt is made to assume a specific target number or target size.

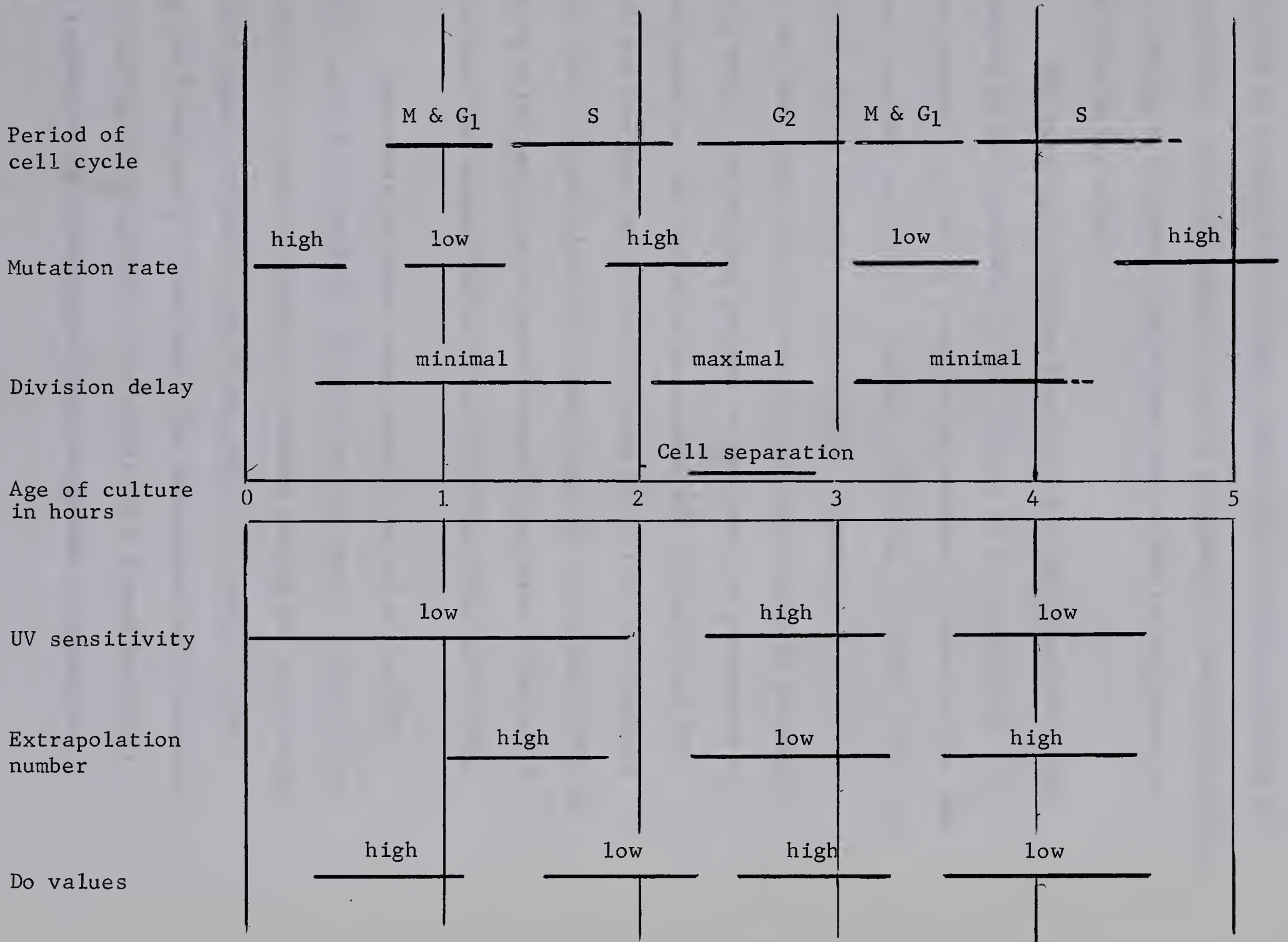
In the analysis that was carried out on survival curves in this work, errors that might result from the changing curvature in the region of reduced survival would not be encountered, as all experimentation was designed to provide survival in the range of 0.5% to 10%. The extrapolation numbers as they are used in this discussion are considered only in respect to their changing magnitude throughout the cell cycle. It is not proposed that these relate directly to a specific target number or size. The changes in the extrapolation numbers might be interpreted to mean that changes do take place in the target, or targets, and that these changes reflect the amount of UV energy required for target inactivation in different stages of the cell cycle.

The pattern of Do values and extrapolation numbers obtained from survival curves of UV-irradiated U. hordei sporidia resembled rather closely the pattern of those obtained from survival curves of X-ray irradiated human cells (Whitmore et al., 1965). The

studies carried out on U. hordei indicated further that the survival pattern, as a response to X-ray irradiation, was similar to the pattern obtained in UV-irradiation studies. This implies that even though the characteristics of the two types of irradiation are different the cellular response to their damage is similar, at least with respect to their survival patterns as related to different stages in the cell cycle.

During counting and replication it was observed that irradiated colonies of culture ages ranging from 2 to 2-3/4 hours were consistently 8 to 12 hours slower in developing than those of treatments that preceded or immediately following this period. As compared with control plates some reduction in growth rate was noted for all culture-age treatment. However, minimal inhibition from the effect of UV irradiation occurred from 0 to 2 hours and again between 3 and 4 hours. DNA synthesis, which apparently takes place just after nuclear separation, occurs between 1 and 2 hours (Unrau 1965). The several responses of U. hordei sporidia to the effects of UV irradiation are shown in Table 8. These responses as functions of culture age are shown as they are related to one another as well as to the different stages of the cell cycle. The M, G_1 and G_2 phases are located on the basis of available evidence relating to the time of DNA synthesis. Though the exact duration of each phase needs to be verified, these approximations will be helpful in considering the UV responses of sporidia as those related to the cell cycle.

Table 8. The several responses of U. hordei sporidia compared to one another, and to the periods of the cell cycle.



M - mitosis; G_1 - interval between mitosis and DNA synthesis;
S - DNA synthesis; G_2 - interval between DNA synthesis and mitosis.

RNA and protein synthesis probably occur throughout the G_2 period. During this time the irradiation-produced division delay is maximal and UV sensitivity is high, particularly at the beginning of the G_2 period. Since UV light of 2537 Å is known to inactivate nucleic acids it might be expected that either DNA or RNA is implicated in the division-delay effect.

DNA segments acting as templates for RNA formation, might be expected to be vulnerable to the effects of UV irradiation. If it were assumed that the UV irradiation produced a lesion in the DNA segment, then RNA and protein synthesis would be suspended until the cell could affect a repair of the damage. Damage in a specific region may mean that only the activity of that region is suspended allowing other synthesizing regions to continue the production of RNA and protein. The period of suspended RNA synthesis may well account for the long periods of division delay that are observed at this time. If RNA alone were inactivated it is unlikely that an extensive delay would occur since the still functional DNA would soon replace the damaged RNA with newly synthesized, normal RNA.

High mutation rates occur toward the end of the DNA synthetic period and probably extend into the early stages of the G_2 period. If protein synthesis is reduced during DNA replication one would expect enzymatic repair mechanisms to operate slowly during the S and early G_2 periods. The slow repair of UV-induced lesions during these periods would result in a larger number of these lesions being incorporated into the genome as mutations.

As the mutation rate drops throughout G_2 one would expect that repair mechanisms are beginning to operate. An examination of Do values for this region of the cell cycle verifies this assumption. Do values increase as the cell advances into G_2. Increasing Do values indicate a decrease in slope which suggests the functioning of repair mechanisms.

The work of Witkin (1963) and Doudney (1963) has indicated that a mutation must be preceded by some inhibitory effect on the DNA in addition to the production of a damaged region. Usually these occur together and in order to fix the mutation, two conditions must be met: (i) RNA and protein synthesis must occur and, (ii) DNA synthesis must take place in order to finally incorporate the mutation into the genome. It appears that all of these conditions occur in the region of the cell cycle where mutation rates are high.

The repair processes that occur in late G_2 might be explained on the basis of the mechanism proposed by Setlow and Carrier (1964) and by Boyce and Howard-Flanders (1964), which involves an excision of the damaged area followed by a replacement of the base sequence. When the damaged DNA is either completely or partially repaired the cellular activities proceed. Possibly, the mutation is fixed in the genome by the DNA synthesis required to effect the repair.

It does not seem that repair mechanisms can be called upon to account for the low mutation rates that occur in the M, G_1 and early S regions of the cell cycle. During this time division delay is minimal, Do values become low and survival increased. The template

role of DNA is probably suspended as the genome condenses in preparation for mitotic separation. In the condensed state the DNA is perhaps "insulated" against a moderate attack of UV irradiation. This is indicated by the presence of large shoulders in the UV-irradiation survival curves for this portion of the cell cycle. With larger doses of UV the survival curves drop off sharply. When a certain critical dose of UV energy has been imposed upon the genome, any additional dosage causes a rapid inactivation and subsequent cell death.

An earlier remark indicated that the high mutation rates obtained might be a function of the effects of synchronization. Cells prepared for synchronized cell division increase in size and although the DNA does not seem to increase, there appears to be considerable protein synthesis. An increase in the protein content per cell might contribute to the rapid fixation of mutants before damages or errors in DNA can be corrected by repair mechanisms. On the other hand, if protein synthesis is extensive in the period during which the sporidia are prepared for synchronized growth, it is quite conceivable that the normal balance in cell components would be considerably altered. The state of imbalance in the cell's synthetic processes might reduce the normal stability of the cell and thus contribute to an increase in the rate of mutation.

Recent work by Gorman et al. (1964) has indicated that the timing of enzyme synthesis in yeasts can be specifically related to the same stage in each synchronized division. The cellular responses to UV irradiation can also be related to a specific region of the

life cycle. A cell's activities and its responses to external influences are probably a reflection of an ordered change that occurs in the transcription mechanism of the cell.

An interpretation of a cell's overall response to irradiation damage must make some attempt to relate the several radiation responses to the normal cell cycle. Since irradiation damage to the cell have been largely associated with damage to the DNA, one ultimately must attempt to interpret the cellular responses in terms of the irradiation effects on DNA.

In studies carried out on both the forward and reverse mutation rates in U. maydis, Ishikawa (1962) concludes that spontaneous mutations are a function of a certain metabolic state of the cell and that this metabolic state might be concerned with DNA. It appears that this is also true for U. hordei where one must also consider the metabolic disturbances introduced into the cell during the UV treatment. Whatever might be the nature of these disturbances, it appears that the DNA is either directly or indirectly affected, and although cell death is constant for each culture-age treatment the mutation rates vary with different stages in the cell cycle. This must mean that the mutation rate is a function of a particular cellular state at the time of irradiation. The UV-introduced alterations in the cells metabolic system bring about interactions within the cell which are different for different stages in the cell cycle. These differences in turn are reflected in the changing pattern of mutation rates throughout the cell cycle. Some

of the mechanisms that might be involved in these interactions were referred to earlier.

As extensive investigations have not been carried out with the arginine mutant, V-441, it is impossible to account for the contradictory results with respect to mutation rates. The several irradiation responses of this mutant parallel those obtained in studies of normal sporidia, with the exception that the times of maximum mutation yield do not agree. High back-mutation rates occur in the region of the cell cycle where forward-mutation rates are lowest. One immediately interprets this difference to be due to a peculiarity of the mutant. Since arginine is closely associated with histone synthesis one might take the high reverse-mutation rate during M, G_1 and early S periods as an indication that the mutant is not able to properly protect itself during this time. The data obtained from reverse mutation studies suggest that one must be cautious in generalizing the results obtained in this kind of an experiment.

Since sporidial cultures of Ustilago hordei can be readily synchronized, this organism promises to be useful in making several investigations. It would be helpful to have more information on the relationship of RNA and protein synthesis with different stages in the cell cycle. Possibly radio-isotopes could be used in clarifying the nature of the repair process. This might be accomplished by carrying out pulse-labelling experiments in combination with either autoradiographical studies or biochemical analyses on both irradiated

and nonirradiated synchronized cultures. A consideration of the synchronization process itself, might provide information that would be helpful in clarifying the nature of cell division.

SUMMARY

1. Irradiation studies were carried out: (1) to produce mutations in Ustilago hordei, and (2) to study the changing pattern of UV sensitivity and mutation rate in synchronized cultures of U. hordei.

2. More than 500 biochemically deficient mutants were produced. Preliminary studies suggest that the parasite remains infective to susceptible host varieties when it is heterozygous for a biochemical deficiency but is noninfective to susceptible host varieties when it is homozygous for a biochemical deficiency.

3. The discovery of a method for synchronizing U. hordei sporidia has provided a useful tool in measuring UV-irradiation responses throughout the cell cycle of the organism. The changing pattern of UV sensitivity suggested that at least two separate mechanisms were involved: one, there were changes in the overall pattern of sensitivity which were related to the changing aspects of DNA activity, and two, there was a change in the pattern of repair activity. A changing pattern of mutation induction was also obtained. The phenomena of cell death and mutation induction by UV irradiation appeared to be associated with changing aspects of the DNA synthetic cycle.

5. Back mutation studies were carried out, using a synchronously dividing culture of mutant V-441 which showed a biochemical deficiency for arginine. High back mutation rates occurred in the region of the cell cycle where forward mutation rates were lowest.

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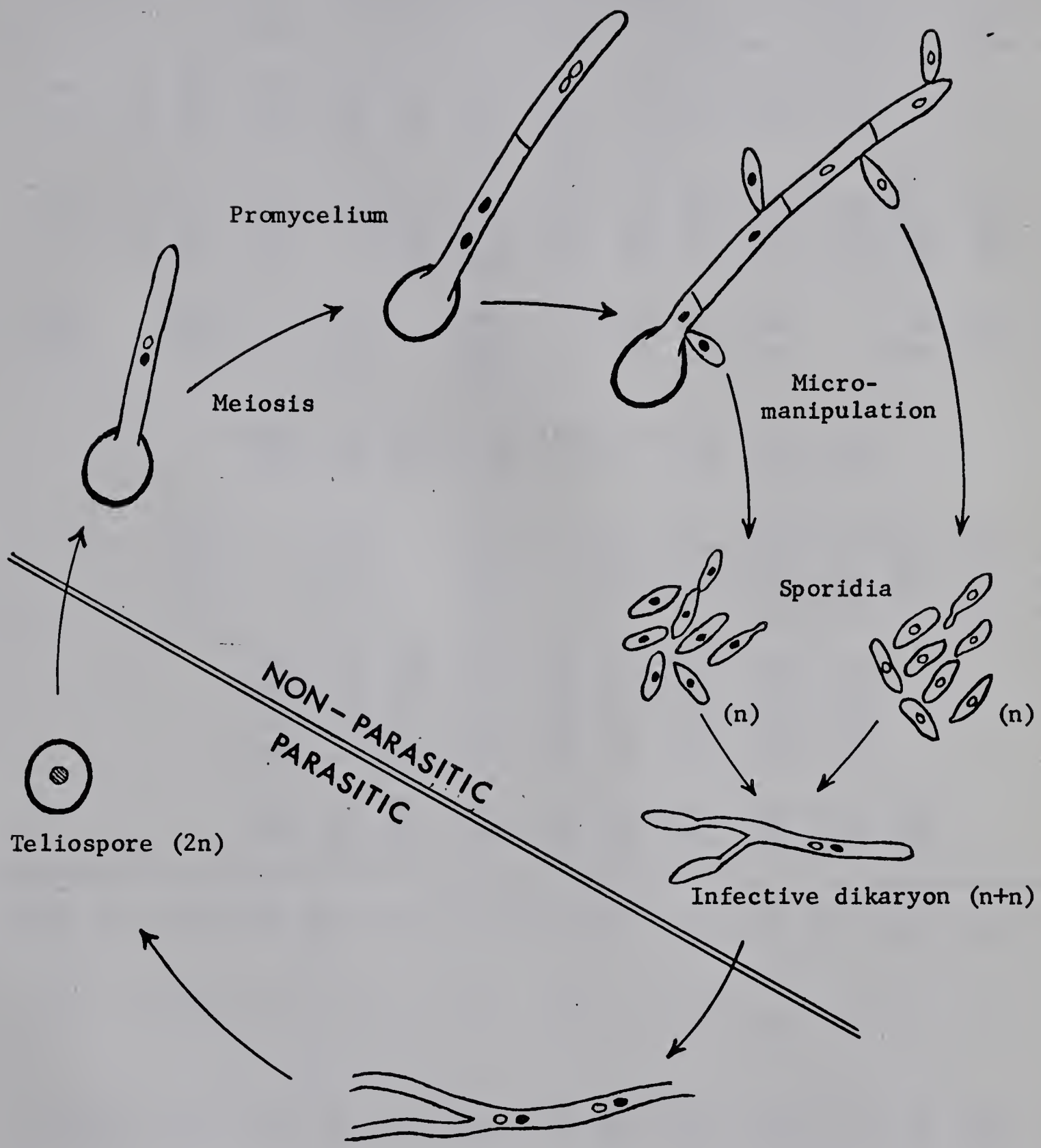
APPENDICES

APPENDIX 1



Life cycle of Ustilago hordei

APPENDIX 2. Synchronization Procedure A. Sporidial numbers in synchronized cultures of U. hordei as measured by colony counts. The values recorded were obtained as controls for the experiments indicated.

Experiment	Age of culture in hours														
	0	1/2	1	1-1/2	2	2-1/2	3	3-1/2	4	4-1/2	5	5-1/2	6	6-1/2	7
IV	144	148	146	149	159	198	252	202	192	203	328	362			
	152	172	147	168	140	180	242	212	223	207	292	342			
	149	169	157	142	158	181	201	220	183						
V	146	104	135	128	141	181	195	217	217	224	346	360	312	404	346
	148	136	146	138	138	170	202	227	220	203	316	352	308	364	370
		141	116	113		182	208	239	237			360	372	374	384
VI	135		145		145		262		223	275	314		308	406	394
	152		135		130		230		146	271	246		396	354	336
					152		257								
VII				134	124	150	175	218	183	185	278	352			
				148	148	182	205	216	210	199	280	306			
VIII			157		138		177		246	210	256	302	368	378	402
			165		167		217		242	234	312	356	336	352	406
			175		152		229		268	253	256	344	384	330	386
			163		142		192		246	265	290	322		372	400
IX				163	129	176	175	213	221	227	264	312			
				155	125	173	233	208	216	184	258	312			
				161	119	169	194	236	269	224	290	298			
				160	152	176	248	199	253	203	310	342			
XI				156	138	170	270	222	260	295	374	454	458		
				147	143	187	250	270	250	294	332	458	478		
Total	1026	870	1787	2062	2840	2475	4614	3099	4505	4156	5342	5937	3720	3334	3424
No. of plates	7	6	12	14	20	14	21	14	20	18	18	17	10	9	9
Average no. of colonies per plate	154	145	149	147	142	177	220	221	225	231	297	349	372	370	380
Concentration cells/ml x 10^6	1.54	1.45	1.49	1.47	1.42	1.77	2.20	2.21	2.25	2.31	2.97	3.49	3.72	3.70	3.80

APPENDIX 3. Synchronization Procedure B. Sporidial numbers in synchronized cultures of *U. hordei* as measured by hemacytometer counts taken at 1/4-hour intervals.

	Age of culture in hours									
	1	1-1/4	1-1/2	1-3/4	2	2-1/4	2-3/8	2-1/2	2-3/4	3
Culture (i)	212	207	219	239	230	227	304	403	454	483
Culture (ii)	181	183	144	207	188	194	249	326	363	386
Culture (iii)	157	135	143	157	145	170	229	269	256	286
Total	549	525	506	603	563	591	782	998	1073	1155
Concentration cells/ml x 10^6	1.10	1.03	1.01	1.21	1.12	1.18	1.57	2.00	2.15	2.32

	Age of culture in hours									
	3-1/4	3-1/2	3-3/4	4	4-1/4	4-1/2	4-3/4	5	5-1/4	5-1/2
Culture (i)	477	448	545	579	686	870	850	858	862	860
Culture (ii)	368	343	392	332	479	638	609	736	736	740
Culture (iii)	291	327	310	338	450	620	595	545	662	619
Total	1136	1108	1247	1249	1615	2128	2054	2139	2260	2219
Concentration cells/ml x 10^6	2.27	2.22	2.50	2.50	3.24	4.26	4.10	4.28	4.52	4.44

APPENDIX 4. Data required to determine percentage synchronization by the method of Engelberg, 1961. Values are derived from the data of Appendix 3.

Age of culture in minutes	Time interval in minutes (dt)	No. of cells which divided (dn)	Mean no. of cells (n)	$R = \frac{dn/dt}{n}$
122.5	120 - 125	0	560	.0000
127.5	125 - 130	10	565	.0035
132.5	130 - 135	40	590	.0136
137.5	135 - 140	90	648	.0278
142.5	140 - 145	190	800	.0475
147.5	145 - 150	100	950	.0210
152.5	150 - 155	50	1020	.0098
157.5	155 - 160	30	1055	.0057
162.5	160 - 165	20	1080	.0037
167.5	165 - 170	14	1095	.0027
172.5	170 - 175	10	1110	.0018
177.5	175 - 180	6	1115	.0009
182.5	180 - 185	0	1120	.0000
187.5	185 - 190	0	1120	.0000

APPENDIX 5. Data required to determine percentage synchronization by the method of Engelberg, 1961. Values are derived from the data of Appendix 3.

Age of culture in minutes	Time interval in minutes (dt)	No. of cells which divided (dn)	Mean no. of cells (n)	$R = \frac{dn/dt}{n}$
217.5	215 - 220	0	1120	.0000
222.5	220 - 225	20	1130	.0035
227.5	225 - 230	34	1156	.0059
232.5	230 - 235	48	1196	.0081
237.5	235 - 240	68	1258	.0102
242.5	240 - 245	96	1330	.0144
247.5	245 - 250	114	1440	.0158
252.5	250 - 255	130	1560	.0167
257.5	255 - 260	144	1700	.0170
262.5	260 - 265	118	1840	.0128
267.5	265 - 270	88	1940	.0091
272.5	270 - 275	68	2020	.0068
277.5	275 - 280	52	2075	.0050
282.5	280 - 285	36	2120	.0034
287.5	285 - 290	30	2152	.0028
292.5	290 - 295	22	2180	.0020
297.5	295 - 300	16	2200	.0015
302.5	300 - 305	12	2210	.0011
307.5	305 - 310	10	2220	.0009
312.5	310 - 315	8	2230	.0007
317.5	315 - 320	6	2235	.0005
322.5	320 - 325	0	2240	.0000

APPENDIX 6. Data obtained from experiments XIV to XVIII in which synchronizing procedure A was followed. In each experiment two dosages of UV irradiation were used to treat cultures of different ages. A series of five partially-overlapping experiments were performed. The % survival, which is exponential, is converted to a linear scale by the use of logarithms. Regression coefficients and Do-values are calculated. Extrapolation numbers and % survival at 4 minutes of UV were estimated after plotting the survival curves on semilogarithmic paper (see text).

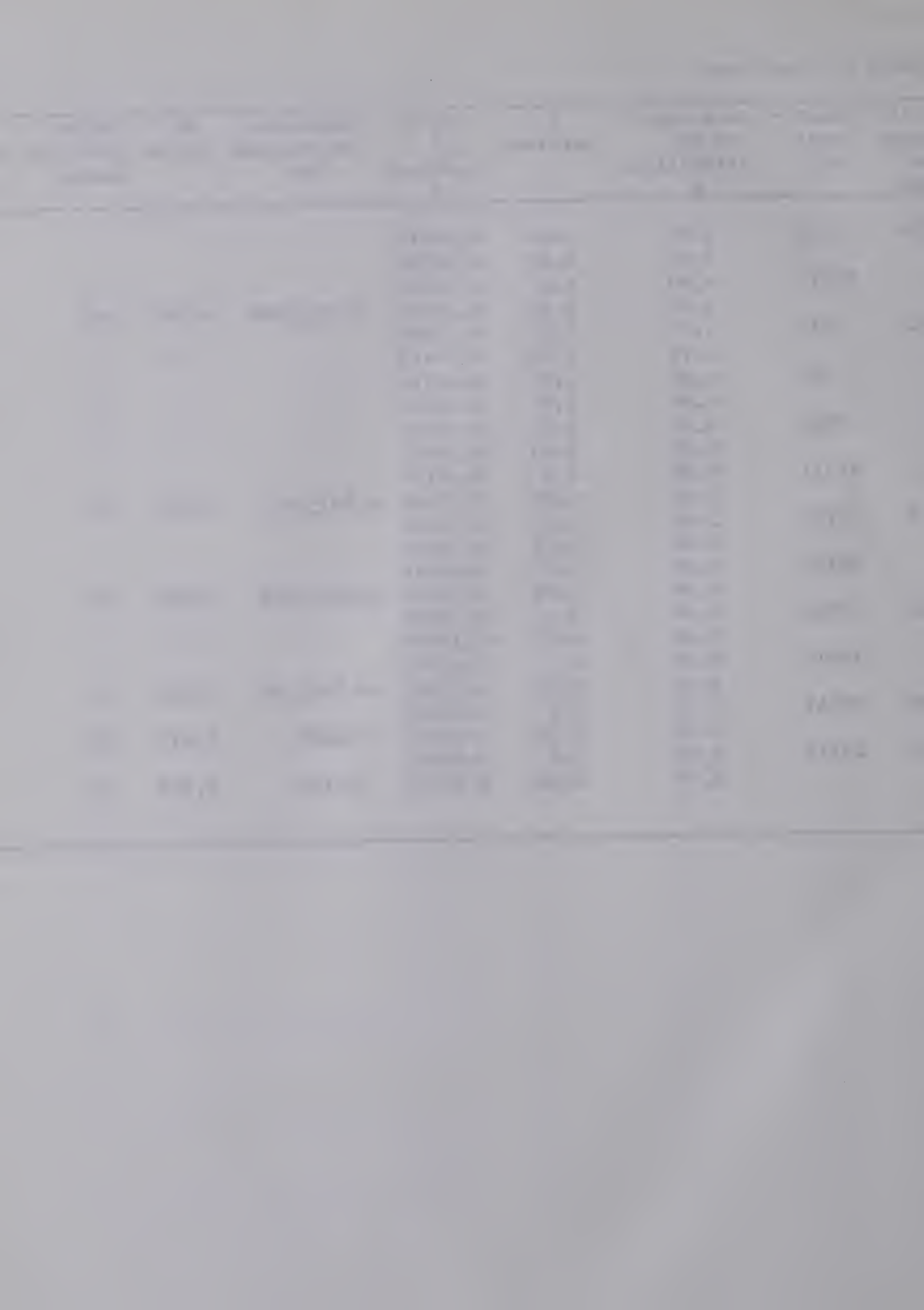
Age of culture in hours	Experiment no.	Dose min. of UV irradiation X	% survival	Log of % survival Y	Regression coefficient DYX	Do value	Extrapolation number	% survival 4 min. UV
1/4	XV	7.50	10.4	1.01703				
		8.00	5.6	0.74819	-0.538	0.803	1000	100
3/4	XV	7.75	6.5	0.81291				
		8.12	4.2	0.62325	-0.513	0.842	480	100
1-1/4	XV	7.25	14.0	1.14613				
		7.75	7.1	0.85126	-0.590	0.732	2200	100
1-3/4	XIV	7.25	1.5	0.17609				
		7.75	0.56	-0.25181				
	XV	6.50	4.3	0.63347				
		6.75	3.0	0.47712	-0.700±.044	0.617	1500	80
2	XVI	5.50	4.0	0.60206				
			0.6	-0.22185	-0.659	0.655	160	35
2-1/8	XVI	5.25	5.8	0.76343				
		6.50	0.69	-0.16115	-0.740	0.584	430	45
2-1/4	XIV	5.25	2.6	0.41497				
		5.75	1.35	0.13033				
	XV	5.00	3.5	0.54407				
		5.50	1.7	0.23045				
	XVI	4.50	12.0	1.07918				
		6.25	0.85	-0.07058	-0.643±.077	0.672	66	18
2-3/8	XVI	4.50	11.0	1.04139				
		5.75	1.82	0.26007	-0.625	0.691	66	25
2-1/2	XVI	4.25	7.6	0.88081				
		5.50	1.68	0.22531	-0.524	0.823	12.5	10
2-5/8	XVI	3.75	11.0	1.04139				
		5.25	2.0	0.30103	-0.494	0.875	7.2	8.2
2-3/4	XIV	4.50	1.3	0.11394				
		5.00	0.73	-0.13668				
	XV	4.25	1.9	0.27875				
		4.75	1.2	0.07918				
	XVI	3.50	8.5	0.92942				
		5.25	1.23	0.08991	-0.538±.127	0.803	4.6	3.3
2-7/8	XVI	3.50	9.4	0.97312				
		5.00	1.6	0.20412	-0.513	0.842	5.7	5.0
3	XVI	3.50	7.5	0.87506				
		5.00	1.1	0.04139	-0.556	0.777	6.4	4.0
3-1/4	XIV	4.25	5.0	0.69897				
		4.75	2.7	0.43136				
	XV	4.00	6.0	0.77815				
		4.50	3.8	0.57978	-0.464±.059	0.931	4.7	6.3

APPENDIX 6. Continued

Age of culture in hours	Experiment no.	Dose min. of UV irradiation X	% survival	Log of % survival Y	Regression coefficient DYX	Do value	Extrapolation number	% survival 4 min. UV
3-3/4	XIV	5.25	8.2	0.91381				
		6.00	2.7	0.43136				
	XV	5.25	7.6	0.88081				
		5.75	3.3	0.51851				
	XVIII	5.75	2.9	0.46240				
		7.00	0.20	-0.69897	-0.899±.067	0.481	4300	70
3-7/8	XVIII	5.50	1.60	0.20412				
		6.75	0.14	-0.85387	-0.846	0.510	660	25
4	XVIII	5.50	4.60	0.66276				
		6.50	0.50	-0.30103	-0.964	0.448	8000	80
4-1/8	XVIII	5.50	1.40	0.14613				
		6.50	0.14	-0.85387	-1.000	0.432	4300	45
4-1/4	XIV	5.75	2.0	0.30103				
		6.25	0.84	-0.07571				
	XV	5.50	3.3	0.51851				
		6.00	1.7	0.23045				
	XVIII	5.50	2.1	0.32222				
		6.50	0.18	-0.74473	-1.009±.151	0.428	12000	80
4-3/8	XVIII	5.25	1.3	0.11394				
		6.25	0.14	-0.85387	-0.968	0.446	1400	20
4-1/2	XVII	5.00	5.0	0.69897				
		6.00	0.74	-0.13077				
	XVIII	5.00	3.50	0.54407				
		6.00	0.39	-0.40894	-0.891±.050	0.484	1200	25
4-5/8	XVII	4.75	3.0	0.47712				
		5.75	0.60	-0.22185				
	XVIII	4.75	2.6	0.41497				
		5.75	0.32	-0.49485	-0.804±.039	0.537	185	1.2
4-3/4	XIV	4.75	2.0	0.30103				
		5.25	0.90	-0.04576				
	XV	4.50	3.5	0.54407				
		5.00	2.0	0.30103				
	XVII	4.50	6.3	0.79934				
		5.75	0.50	-0.30103				
	XVIII	4.50	4.2	0.62325				
		5.75	0.34	-0.46852	-0.824±.025	0.524	210	11
4-7/8	XVII	4.25	3.0	0.47712				
		5.50	0.50	-0.30103				
	XVIII	4.25	4.3	0.67347				
		5.50	0.50	-0.30103	-0.701±.025	0.616	370	4.2
5	XVII	4.00	10.0	1.00000				
		5.25	0.63	-0.20066				
	XVIII	4.25	5.2	0.71600				
		5.25	0.82	-0.08619	-0.896±.057	0.482	78	11

APPENDIX 6. Continued

Age of culture in hours	Experiment no.	Dose min. of UV irradiation X	% survival	Log of % survival Y	Regression coefficient DYX	Do value	Extrapolation number	% survival 4 min. UV
5-1/8	XVII	4.00	5.0	0.69897				
		5.25	0.65	-0.18709				
	XVIII	4.00	4.8	0.68124				
		5.25	0.55	-0.25964	-0.731±.030	0.591	42	5.6
5-1/4	XIV	4.25	1.5	0.17609				
		4.75	0.57	-0.24413				
	XV	4.00	2.8	0.44716				
		4.50	1.4	0.14613				
	XVII	3.75	8.0	0.90309				
		5.25	0.53	-0.27572				
	XVIII	4.00	4.8	0.68124				
		5.25	0.28	-0.55284	-0.837±.011	0.516	78	4.8
5-3/8	XVII	3.75	5.4	0.73239				
		5.00	0.72	-0.14267				
	XVIII	4.00	5.0	0.69897				
		5.00	0.76	-0.11019	-0.734±.068	0.589	37	4.2
5-1/2	XVII	3.50	8.5	0.92942				
		5.00	0.65	-0.18709				
	XVIII	4.00	4.5	0.65321				
		5.00	0.60	-0.22185	-0.779±.047	0.555	49	3.7
5-5/8	XVIII	3.75	5.2	0.71600				
		5.00	0.38	-0.42022	-0.909	0.475	120	2.9
5-3/4	XVIII	4.00	3.5	0.54407				
		5.00	0.56	-0.25181	-0.796	0.543	52	3.5



APPENDIX 7. Data obtained from experiments XIX and XX in which synchronizing procedure B was followed. In each experiment two dosages of UV irradiation were used to treat cultures of different ages. The % survival, which is exponential, was converted to a linear scale by the use of logarithms. Regression coefficients and Do values were calculated. Extrapolation numbers and % survival at 4 minutes of UV were estimated after plotting the survival curves on semilogarithmic paper (see text).

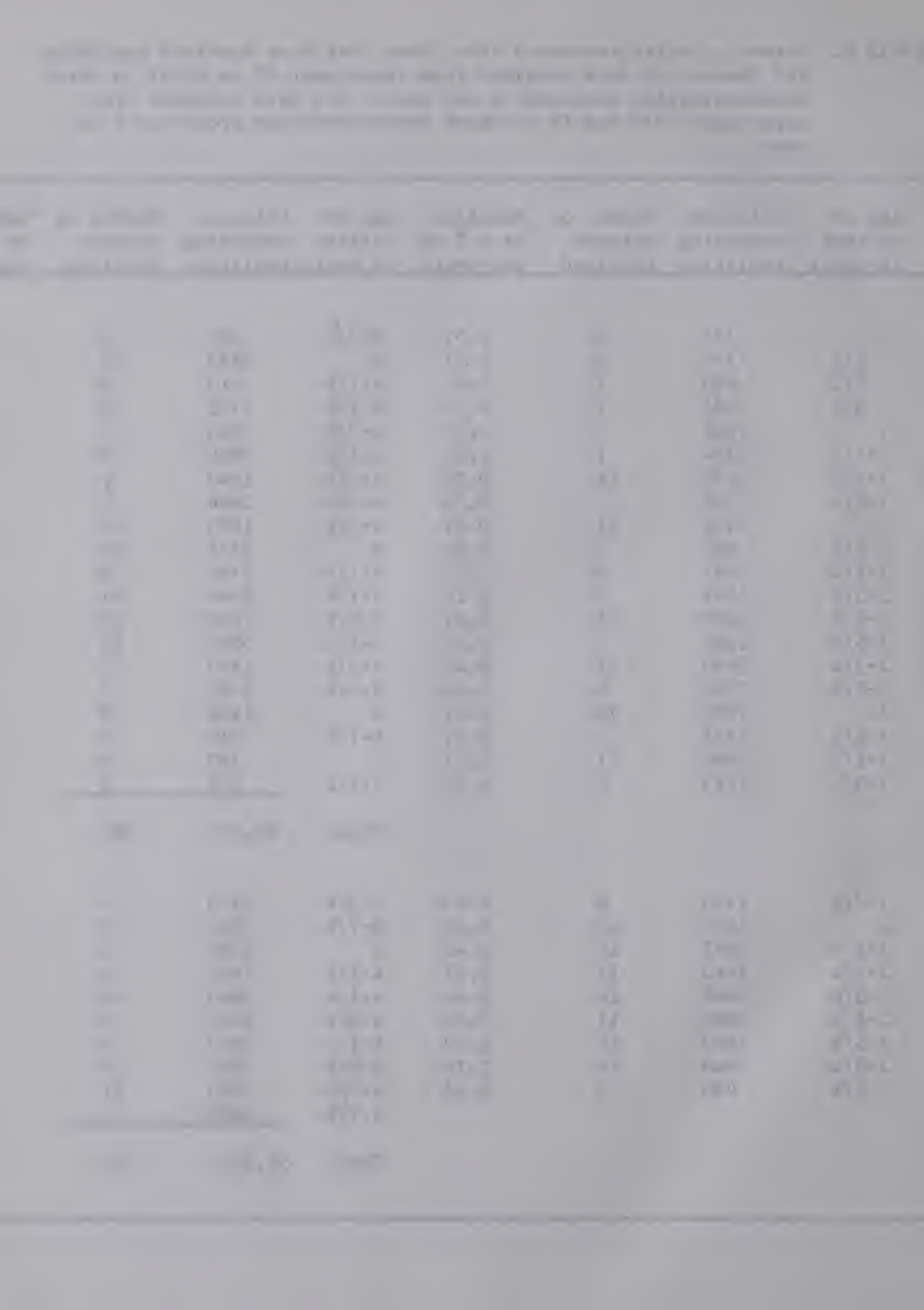
Age of culture in hours	Trial no.	Dose min. of UV irradiation X	% survival	Log % survival Y	Regression coefficient byx	Do values	Extra-polation number	% survival 4 min. UV
1-7/8	XIXA	5.00	17.6	1.24551				
		6.00	4.4	0.64345	-0.602	0.717	180	65
2	XIXB	4.50	26.0	1.41497				
		5.50	5.6	0.74819				
	XXA	5.50	5.6	0.74819				
		6.75	0.87	-0.06048	-0.655	0.659	220	50
2-1/8	XIXA	4.75	20.0	1.30103				
		5.75	4.8	0.68124				
	XXB	5.25	10.7	1.02938				
		6.50	1.4	0.14613	-0.666	0.648	320	65
2-1/4	XIXB	4.00	23.0	1.36173				
		5.25	5.2	0.71600				
	XXA	5.00	7.1	0.85126				
		6.25	0.90	-0.04576	-0.625	0.691	82	26
2-3/8	XIXA	4.00	8.90	0.94939				
		5.25	1.80	0.25527				
	XXB	4.25	9.10	0.95904				
		5.50	1.80	0.25527	-0.537	0.804	15	10
2-1/2	XIXB	4.00	6.50	0.81291				
		5.25	1.70	0.23045				
	XXA	4.00	8.00	0.90309				
		5.50	1.30	0.11394	-0.498	0.867	7.0	7.2
2-5/8	XIXA	3.75	7.00	0.84510				
		5.25	1.40	0.14613				
	XXB	4.00	5.00	0.69897				
		5.50	1.00	0.00000	-0.469	0.920	3.9	5.2
2-3/4	XXA	4.50	3.10	0.49136				
		6.00	0.58	-0.23657	-0.485	0.889	4.8	5.3
2-7/8	XXB	5.00	1.80	0.25527				
		6.25	0.50	0.30103	-0.445	0.970	3.1	5.0
3-3/4	XIXA	5.00	12.5	1.09691				
		6.00	2.4	0.38021	-0.717	0.602	500	60
3-7/8	XIXB	5.00	12.5	1.09691				
		6.00	2.1	0.32222	-0.774	0.557	1100	70
4	XIXA	4.75	9.4	0.97313				
		5.75	1.7	0.23045				
	XXB	4.75	19.0	1.27875				
		5.75	3.5	0.54407	-0.739	0.585	440	56

APPENDIX 7. Continued

Age of culture in hours	Trial no.	Dose min. of UV irradiation X	% survival	Log % survival Y	Regression coefficient byx	Do values	Extra-polation number	% survival 4 min. UV
4-1/8	XIXB	4.75	9.2	0.96379				
		5.75	2.2	0.34242				
	XXA	4.75	13.6	1.13354				
		5.75	2.1	0.32222	-0.716	0.602	300	38
4-1/4	XIXA	4.50	6.3	0.79934				
		5.25	1.5	0.17609				
	XXB	4.50	16.6	1.22011				
		5.25	4.2	0.62325	-0.813	0.531	540	25
4-3/8	XIXB	4.25	8.0	0.90309				
		5.25	1.6	0.20412				
	XXA	4.25	12.1	1.08279				
		5.25	2.9	0.46240	-0.660	0.655	80	14
4-1/2	XIXA	4.25	4.7	0.67210				
		5.25	1.1	0.04139				
	XXB	4.25	10.2	1.00860				
		5.25	2.0	0.30103	-0.669	0.645	50	10
4-5/8	XIXB	4.00	5.4	0.73239				
		5.00	1.2	0.07918				
	XXA	4.00	8.4	0.92428				
		5.00	1.7	0.23045	-0.674	0.641	35	6.9
4-3/4	XIXA	4.00	7.0	0.84510				
		5.00	1.3	0.11394				
	XXB	4.00	7.6	0.88081				
		5.00	1.3	0.11394	-0.749	0.576	80	7.3
4-7/8	XIXB	4.25	4.7	0.67210				
		5.25	0.70	-0.15490	-0.827	0.522	135	7.3

APPENDIX 8. Mutant colonies recovered from those that have survived radiation. (a) Summary of data obtained from experiment IV to XVIII in which synchronization procedure A was used. (b) Data obtained from experiment XIX and XX in which synchronization procedure B was used.

	Age of culture in hours	Colonies surviving radiation	Number of mutants obtained	Mutation as a % of survivors	Age of culture in hours	Colonies surviving radiation	Number of mutants obtained	Mutation as a % of survivors
(a)								
	0	701	4	0.57	3-7/8	250	0	-
	1/4	1376	3	0.22	4	3933	13	0.33
	1/2	830	7	0.84	4-1/8	210	0	-
	3/4	985	1	0.10	4-1/4	1772	8	0.45
	1	1324	3	0.23	4-3/8	240	1	0.42
	1-1/4	2304	1	0.04	4-1/2	3880	17	0.44
	1-1/2	3672	14	0.38	4-5/8	1040	9	0.87
	1-3/4	1758	5	0.28	4-3/4	3868	9	0.23
	2	5418	21	0.39	4-7/8	1670	14	0.84
	2-1/8	682	3	0.44	5	5271	25	0.48
	2-1/4	2197	6	0.27	5-1/8	2330	8	0.34
	2-3/8	1539	3	0.20	5-1/4	4588	14	0.31
	2-1/2	4090	25	0.61	5-3/8	2650	15	0.57
	2-5/8	2140	7	0.33	5-1/2	5390	24	0.45
	2-3/4	2430	11	0.45	5-5/8	1310	2	0.15
	2-7/8	2266	4	0.18	5-3/4	1100	7	0.63
	3	7039	26	0.37	6	1408	3	0.21
	3-1/4	4373	3	0.07	6-1/2	959	4	0.42
	3-1/2	5250	11	0.21	7	760	3	0.40
	3-3/4	4113	6	0.15	7-1/2	276	3	1.10
					Total	97,392	343	
(b)								
	1-7/8	1333	6	0.45	3-3/4	1950	2	0.10
	2	4192	13	0.35	3-7/8	2520	2	0.08
	2-1/8	3827	16	0.42	4	4300	5	0.12
	2-1/4	4110	11	0.27	4-1/8	4480	6	0.14
	2-3/8	3496	16	0.46	4-1/4	4890	10	0.22
	2-1/2	3290	11	0.33	4-3/8	4920	6	0.12
	2-5/8	2490	12	0.48	4-1/2	3640	5	0.14
	2-3/4	840	10	1.19	4-5/8	3280	10	0.34
	2-7/8	480	3	0.63	4-3/4	3920	11	0.28
					4-7/8	1400	2	0.14
					Total	59,358	157	



APPENDIX 9. Data on irradiation and survival obtained with arginine mutant No. V-441. Synchronized procedure B was followed. The regression coefficients and Do-values were calculated. Extrapolation numbers and the % survival at 4 minutes of UV irradiation were estimated after survival curves were plotted on semilogarithmic paper (see text).

Age of culture in hours	Dose min. of UV irradiation X	% survival	Log % survival Y	Regression coefficient byx	Do values	Extrapolation numbers	% survival
.6	7.75	34.0	1.53148				
	8.75	17.0	1.23045	-0.301	1.434	70	100
1.0	7.75	33.0	1.51851				
	8.75	17.0	1.23045	-0.288	1.499	55	100
1.3	7.50	29.0	1.46240				
	8.50	17.0	1.23045	-0.232	1.861	16	100
1.6	7.00	21.0	1.3222				
	8.00	7.5	0.87506	-0.447	0.965	260	100
1.9	6.25	17.4	1.24055				
	7.25	5.4	0.73239	-0.508	0.850	260	95
2.2	5.00	29.0	1.46240				
	6.00	10.2	1.00860	-0.454	0.952	52	60
2.6	4.00	12.0	1.07918				
	5.25	5.7	0.75587	-0.259	1.664	1.3	12
2.9	4.00	7.0	0.84510				
	5.25	2.7	0.43136	-0.332	1.301	1.5	7
3.2	5.75	2.6	0.41497				
	6.75	1.2	0.07918	-0.336	1.286	2.1	10
3.5	7.00	2.5	0.39794				
	8.00	0.65	-0.18709	-0.585	0.738	260	80
3.8	6.00	11.0	1.04139				
	7.00	3.6	0.55630	-0.485	0.890	86	75
4.2	4.50	30.0	1.47712				
	5.50	13.4	1.12710	-0.350	1.234	11	45
4.6	4.50	23.0	1.36173				
	5.75	7.7	0.86649	-0.396	0.872	12	35

APPENDIX 10. Data obtained on the reverse mutation rate of an unstable arginine mutant No. V-441. Cultures were synchronized using procedure B.

Age of culture in hours	Minutes of UV irradiation	% survival	Non irradiated controls **No. of colonies per plate Complete diln 10^{-4}	Non irradiated controls **No. of colonies per plate Minimal diln 10^{-2}	Non irradiated controls Reverse mutation rate $X10^{-4}$	Irradiated material **No. of colonies per plate Complete diln 10^{-3}	Irradiated material **No. of colonies per plate Minimal diln 10^{-1}	Irradiated material Reverse mutation rate $X10^{-3}$
Experiment XXI								
1.6	6.50	16.8	104	7.5	7.2	175	386	22.0
	7.50	7.0				72	225 (4)	31.2
2.0	5.75	14.3	99	10.5	10.6	143	115	8.1
	6.30	6.6				66	112 (4)	17.0
2.4	4.25	8.8	175	10.5	6.0	154	35	2.3
	5.30	2.7				47	30 (4)	6.4
2.8	4.00	9.5	195	15.0	7.7	186	43	2.3
	5.25	4.6				90	32 (4)	3.6
3.2	4.25	17.0	200	15.0	7.5	338	225	6.7
	5.30	8.0				156	310 (4)	19.9
Experiment XXII								
0.2	7.75	41.0	161	3.5	2.2	661	564	8.5
	8.75	21.0				337	366 (4)	10.9x
0.6	7.75	34.0	191	4.5	2.4	664	524	7.9
	8.75	17.0				337	430 (4)	12.8x
1.0	7.75	33.0	154	3.5	2.3	503	535	10.6
	8.75	17.0				267 (3)	438 (4)	16.4x
1.3	7.50	29.0	133	10.0	7.5	386	725	19.1
	8.50	17.5				225 (3)	540 (4)	24.0x
1.6	7.00	21.0	131	16.0	12.2	278	505	18.2x
	8.00	7.5				98 (3)	320 (3)	32.6
1.9	6.25	17.4	123	14.0	11.4	214	263	12.3x
	7.25	5.4				69 (3)	192 (5)	27.8
2.2	5.00	29.0	128	11.0	8.6	372	84	2.2
	6.00	10.2				131 (3)	74 (5)	5.6x
2.6	4.00	12.0	180	11.5	6.4	218	48	2.2
	5.25	5.7				103 (3)	34 (5)	3.3x
2.9	4.00	7.0	189	14	7.4	132 (3)	24	1.8
	5.25	2.7				50 (4)	24 (5)	4.8x
3.2	5.75	2.6	207	14.5	7.0	58	148	25.5x
	6.75	1.2				26 (3)	94 (4)	36.2
3.5	7.00	2.5	180	12	6.7	44	200	45.5x
	8.00	0.65				12 (3)	73 (5)	60.8
3.8	6.00	11.0	192	16.5	8.6	211	374	17.7
	7.00	3.6				65	283 (3)	43.6x
4.2	4.50	30.0	189	13.5	7.1	575	250	4.4
	5.50	13.4				225	328	14.6x
4.6	4.50	23.0	217	16.5	7.6	495	143	2.9
	5.75	7.7				167 (3)	179 (4)	10.4x

x Values used in the graphical representation of relationships between age of culture and reversion rate.

** The number of colones are averages of two plates except where indicated by bracketed numbers.